WHERE TO INVEST? CAUSES AND CONSEQUENCES OF TRADE-OFFS IN THE POLYMORPHIC BLACK SCAVENGER FLY *SEPSIS THORACICA*

Dissertation

Zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Juan Pablo Busso

aus

Argentinien & Italien

Promotionskomitee

Prof. Dr. Wolf U. Blanckenhorn

(Leitung der Dissertation und Vorsitz)

Prof. Dr. Barbara König

PD Dr. Josh Van Buskirk

CONTENTS

SUMMARY

The limiting nature of resources requires organisms to carefully distribute their investment into a variety of traits, resulting in life-history trade-offs. Trade-offs originate because the best phenotype for one task is usually not the best for other tasks. In the black scavenger fly Sepsis thoracica (Robineau-Desvoidy, 1830; Diptera: Sepsidae), males exhibit extreme variability in size and color, with smaller males being mostly black and the largest males bright orange, while females are completely black. This variation is a largely environmentally induced polyphenism. This variation suggests a trade-off between size and melanism in the males but not the females. My dissertation approaches the subject by employing multiple perspectives encompassing several fitness components that influence the phenotypic landscape of the species. Among the components potentially influencing the trade-off, in this thesis I analyze the effects of climatic variables, immunity, sexual selection and predation. I thus focused on the factors most strongly influencing the body size and melanism of the fly. For the five studies reported I raised flies stemming from 4 to 15 European populations ranging from southern Italy to Estonia. All experiments revealed that male Sepsis thoracica feature a sigmoid trade-off between melanism and body size, defining two strikingly different male morphs: obsidian (small and black) and amber (large and yellow). The sigmoid trade-off relationship was altered by the food (= dung) availability and developmental temperature, evincing its plasticity (Chapter 1 & 5). The relationship further evolved across populations in response to the environmental characteristics of their habitat, notably temperature, insolation and UV radiation, suggesting that plasticity also has an underlying genetic basis (Chapter 1). However, results suggest that the plastic and evolutionary responses to temperature (and other climatic factors) are not identical, so plasticity does not necessarily follow the direction of evolution of the trade-off, but rather adds to it. Moreover, the sigmoid trade-off resulted in obsidian males having better immunity than the ambers (Chapters 4 & 5), whereas the latter showed greater mating success and survival against different predators (Chapters 2 & 3). Overall, this plasticity in resource allocation results in almost discrete alternative phenotypes that represent complementary life-history advantages. While the small obsidians are likelier to survive pathogenic infections, which is particularly important in their pathogen rich environment (dung) but remains to be directly tested, ambers are likelier to survive predation events and to acquire mates once adult. Although each male morph has the clear selective advantages just described depending on the circumstances, I also found disruptive sexual selection acting on the male body size (Chapter 2), which could probably be the force driving the origin and/or at least the maintenance of this rare discrete male polymorphism in the sepsid fly S. thoracica.

ZUSAMMENFASSUNG

In der Natur stets begrenzte natürliche Ressourcen zwingen auch Organismen, die vorhandene Energie auf diverse Merkmale bzw. Tätigkeiten zu verteilen. Dies erfordert permanente Güterabwägungen, engl. Trade-off genannt, denn der beste Phänotyp für eine Funktion ist meist nicht der beste für eine andere. Männchen der ameisenähnlichen Schwingfliege Sepsis thoracica (Robineau-Desvoidy, 1830; Diptera: Sepsidae) unterscheiden sich drastisch in ihrer Körpergrösse und Färbung. Während Weibchen stets schwarz- glänzend sind, sehen kleine Männchen (hier „Obsidians“ genannt) genauso aus, während grosse Männchen orangefarben sind („Ambers“). Dieser Unterschied ist hauptsächlich umweltbedingt, ein sog. Polyphänismus, und suggeriert eine Güterabwägung zwischen Grösse und Färbung. In meiner Dissertation habe ich von diversen Gesichtspunkten aus die Vor- und Nachteile dieser beiden männlichen Merkmalskombinationen untersucht. Dabei konzentrierte ich mich auf klimatische Umweltfaktoren, Immunkompetenz, sexuelle Selektion (d.h. Paarungserfolg), und Prädation, allesamt Faktoren, welche Körpergrösse, Färbung und letztlich die Fitness der Merkmalsträger stark beeinflussen sollten.

Die in den insgesamt fünf Studien verwandten Fliegen entstammten zwischen 4 und 15 europäischen Populationen von Süditalien bis Estland. Sämtliche Studien ergaben einen sigmoiden Zusammenhang (Trade-off) zwischen Männchengrösse und –Färbung, welcher allerdings im Detail (Lage, Steigung, etc.) plastisch von Umweltbedingungen wie der Temperatur oder auch der Nahrungsmenge (Dung) abhängt (Kapitel 1 & 5). Des weiteren zeigten sich z.T. subtile genetische Unterschiede zwischen den Populationen, wie auch Einflüsse der aus Datenbanken extrahierten Klimafaktoren des Herkunftsortes der Fliegen auf die Plastizität des Trade-offs (insbesondere mittlere Temperatur, Sonneneinstrahlung und UV-Strahlung). Dabei stimmten die plastischen, durch Umwelteinflüsse bedingten und die mutmasslich evolvierten geographischen Unterschiede nicht unbedingt überein, weshalb der plastischen Antwort ein Zusatznutzen gegenüber der genetischen Antwort zugeschrieben werden kann (Kapitel 1). Darüber hinaus konnte ich belegen, dass die kleinen schwarzen Männchen (Obsidians) eine bessere Immunkompetenz aufweisen (Kapitel 4 & 5), während die grossen orangefarbenen (Ambers) Männchen höheren Paarungserfolg (Kapitel 2) und auch bessere Überlebenschancen gegenüber wirbellosen Räubern (Spinnen, Fliegen, Wanzen) aufweisen (Kapitel 3). Während die Obsidians aufgrund höherer Phenoloxidasewerte (ein Enzym, das sowohl die schwarze Färbung wie auch die Immunkompetenz bedingt) vermutlich Parasiten besser widerstehen können – was in ihrem Dunghabitat sicher von grosser Wichtigkeit ist, jedoch hier nicht direkt getestet wurde – können Ambers Räubern besser entkommen und sich leichter paaren. Offenbar führen diese evolutionären Vor- und Nachteile, in Kombination mit der phänotypisch-plastischen Reaktionsmöglichkeit in Bezug auf die jeweils vorherrschenden Umweltbedingungen, insgesamt zur Evolution nahezu diskreter, sich ergänzender Phänotypen, eben einem Trade-off. Obgleich jeder der beiden Morphotypen klare (lineare) selektive Vorteile zu haben scheint, habe ich im Kapitel 2 auch gleichzeitig auftretende (nicht-lineare) disruptive Selektion auf die Körpergrösse feststellen können. Theoretisch kann diese ausreichen, um den beschriebenen Dimorphismus der Männchen der Schwingfliege S. thoracica zu erklären, doch dies kann im Nachhinein natürlich nicht bewiesen werden.

GENERAL INTRODUCTION

The limiting nature of resources requires organisms to carefully distribute their investment into a variety of traits, resulting in life-history trade-offs (Zera and Harshman, 2001). Trade-offs at the phenotypic level are very important because their costs are paid in fitness terms, the ultimate common currency in evolutionary biology (Stearns, 1989). Since natural selection acts on phenotypes, phenotypic trade-offs shape the covariation patterns on which selection can act. Trade-offs originate because the best phenotype for one particular task is usually not the best for every task (Campbell et al., 1991). Changes in one trait to improve a fitness component, e.g. survival, can be detrimental on another fitness component, e.g. offspring number (fecundity). Accordingly, organisms tend to maximize their fitness through multi-objective optimization (Abraham and Jain, 2005; Shoval et al., 2012), which not necessarily results in merely one optimal solution (Farnsworth and Niklas, 1995).

An excellent candidate species to analyze trade-offs is the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae), which belongs to a group of small (3 – 8 mm in length) flies with an ant-like habitus that are common worldwide and often breed in rotting plant matter or vertebrate excrements (Pont and Meier, 2002). *Sepsis thoracica* is the only European *Sepsis* that presents a bright orange coloration, while all European close relatives are black (Pont and Meier, 2002). Males of this species exhibit extreme variability in size and color, with smaller individuals being mostly black and large individuals bright orange (Pont and Meier, 2002). These highly correlated gradients suggest a trade-off between body size and melanism. Both body size (Reichle, 1968) and pigmentation are resource-demanding traits, as melanin is costly to synthesize (Talloen et al., 2004), and its synthesis further produces cytotoxic by-products (González-Santoyo and Córdoba-Aguilar, 2012; Schmid-Hempel, 2003). Individuals will therefore need to balance the allocation of resources between body size and melanism to optimize their phenotype and maximize their fitness.

Because of its wide European range, *S. thoracica* allows the comparison of different populations along a vast latitudinal gradient. Along this gradient, trade-offs presumably mediate adjustments to varying thermal and insolation profiles, especially in small ectotherms, whose body temperature follows environmental temperatures closely (Buckley et al., 2012). Two traits normally involved in latitudinal adaption are body size (Chown and Gaston, 2010; Partridge and Coyne, 1997) and melanism (reviewed by Clusella Trullas et al., 2007). Since both traits are very costly, individuals need to optimize the investment balance into each trait. In **Chapter 1** I addressed how environmental factors influence this trade-off in the short and long term.

The fact that the trade-off between body size and melanism is only present in *S. thoracica* males pointed to sexual selection as an important factor influencing it. Body size is one of the most important traits often favored by classic male–male competition and/or female choice (reviewed by Andersson, 1994; Blanckenhorn, 2000; Fairbairn et al., 2007; Hunt et al., 2009). Coloration equally plays an important role in sexual selection (Andersson, 1994; Lozano, 1994). Coloration can affect male-male competition when it works as a signal to other males (Pärt and Qvarnström, 1997; Pérez I de Lanuza et al., 2013), and it can also be a signal for females (Kodric-Brown, 1985; Lozano, 1994), or simultaneously for both males and females (Kodric-Brown, 1996; Pérez I de Lanuza et al., 2013). In **Chapter 2,** by analyzing both male and female behaviors during mating encounters, I investigated how the reproductive fitness of the individuals was affected by the body size and coloration trade-off. I also analyzed whether and if so which mechanism of sexual selection contributes to the maintenance of this trade-off in males.

Besides the reproductive component of fitness, I also analyzed aspects of the survival component. Hence, later chapters are dedicated to the analyses of two central elements affecting survival: predation and immunity. In **Chapter 3** I focus on predation, since it is a major factor affecting the survival of most organisms in nature, thus shaping trade-offs and a species' morphospace (reviewed by Ruxton et al., 2004). Two of the main traits drastically influencing the survival probability of prey individuals are, again, their body size (Berger et al., 2006; Whitman and Vincent, 2008) and coloration (Svanbäck and Eklöv, 2011). Among insects, a widespread strategy to intraspecifically alter coloration is through changes in the melanin production (Cook and Saccheri, 2013; True, 2003), often resulting

in camouflage or the opposite, aposematism (Guilford, 1990). Consequently, in **Chapter 3** I not only analyze the effect of predation on the trade-off between body size and melanism, but also attempt to distinguish the role of coloration for aposematism and/or camouflage. I focused on invertebrate predators because they can have stronger effects than vertebrates especially on small insects (Fagan and Hurd, 1994; Kristensen, 1994; Lang et al., 1999; Wooster, 1994).

The widespread melanic coloration variation, as seen in *S. thoracica*, is in many cases linked to the insect's immunity thanks to the involvement of melanin in the phenoloxidase cascade (Wilson *et al.* 2002; Cotter *et al.* 2004)species that encounter large fluctuations in population density are predicted to exhibit plasticity in their immune system, such that investment in costly immune defences is adjusted to match the probability of exposure to parasites and pathogens (i.e. density-dependent prophylaxis. Melanin is an important component of the cellular immune response mediating the encapsulation of intruders (González-Santoyo and Córdoba-Aguilar, 2012; Iwanaga and Lee, 2005). Hence, the trade-off between body size and melanism also implies a trade-off between body size and immunity. Since a large part of the sepsid life-cycle (egg, larval stage, pupa, adult feeding and mating) occurs in and on a pathogen-rich environment (dung pats), I decided in **Chapter 4** to analyze how body size influences the investment into immunity. And lastly, in **Chapter 5**, in collaboration with a Master student, we closed the circle to link immunity with developmental temperature (as addressed in Chapter 1), by analyzing the effect of temperature on the body size / immunity trade-off.

In its entirety, my dissertation takes an integrative approach in addressing the causes and consequences of the trade-off between body size and melanism in the strikingly polymorphic *S. thoracica*. The encompassing nature of the work sheds light onto this phenomenon from multiple perspectives, to discover the complex network of elements that simultaneously affects phenotypic trade-offs in nature.

REFERENCES

Abraham A, Jain L, 2005. Evolutionary Multiobjective Optimization. In: Abraham A, Jain L, Goldberg R, editors. Evolutionary Multiobjective Optimization: Theoretical Advances and Applications London: Springer London. p. 1-6.

Andersson B, 1994. Sexual Selection: Princeton University Press.

Berger D, Walters R, Gotthard K, 2006. What keeps insects small?—Size dependent predation on two species of butterfly larvae. Evol Ecol 20:575-589.

Blanckenhorn WU, 2000. The evolution of body size: What keeps organisms small? Q Rev Biol 75:385-407.

Buckley LB, Hurlbert AH, Jetz W, 2012. Broad-scale ecological implications of ectothermy and endothermy in changing environments. Global Ecology and Biogeography 21:873-885.

Campbell B, Grime J, Mackey J, 1991. A trade-off between scale and precision in resource foraging. Oecologia 87:532-538.

Chown SL, Gaston KJ, 2010. Body size variation in insects: a macroecological perspective. Biological Reviews 85:139-169.

Clusella Trullas S, van Wyk JH, Spotila JR, 2007. Thermal melanism in ectotherms. Journal of Thermal Biology 32:235-245.

Cook L, Saccheri I, 2013. The peppered moth and industrial melanism: evolution of a natural selection case study. Heredity 110:207-212.

Fagan WF, Hurd L, 1994. Hatch density variation of a generalist arthropod predator: population consequenes and community impact. Ecology:2022-2032.

Fairbairn DJ, Blanckenhorn WU, Székely T, 2007. Sex, size and gender roles: evolutionary studies of sexual size dimorphism: Oxford University Press.

Farnsworth KD, Niklas KJ, 1995. Theories of Optimization, Form and Function in Branching Architecture in Plants. Functional Ecology 9:355-363.

González-Santoyo I, Córdoba-Aguilar A, 2012. Phenoloxidase: a key component of the insect immune system. Entomologia Experimentalis et Applicata 142:1-16.

Guilford T, 1990. The evolution of aposematism. Insect defenses: adaptive mechanisms and strategies of prey and predators:23-61.

Hunt J, Breuker CJ, Sadowski JA, Moore AJ, 2009. Male–male competition, female mate choice and their interaction: determining total sexual selection. Journal of evolutionary biology 22:13-26.

Iwanaga S, Lee B-L, 2005. Recent advances in the innate immunity of invertebrate animals. BMB Reports 38:128-150.

Kodric-Brown A, 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 17:199-205.

Kodric-Brown A, 1996. Role of male-male competition and female choice in the development of breeding coloration in pupfish (*Cyprinodon pecosensis*). Behavioral Ecology 7:431-437.

Kristensen CO, 1994. Investigations on the natural mortality of eggs and larvae of the large white *Pieris brassicae* (L.) (Lep., Pieridae). Journal of Applied Entomology 117:92-98.

Lang A, Filser J, Henschel JR, 1999. Predation by ground beetles and wolf spiders on herbivorous insects in a maize crop. Agriculture, ecosystems & environment 72:189-199.

Lozano GA, 1994. Carotenoids, Parasites, and Sexual Selection. Oikos 70:309-311.

Pärt T, Qvarnström A, 1997. Badge size in collared flycatchers predicts outcome of male competition over territories. Animal Behaviour 54:893-899.

Partridge L, Coyne JA, 1997. Bergmann's rule in ectotherms: is it adaptive? Evolution 51:632-635.

Pérez I de Lanuza G, Font E, Monterde JL, 2013. Using visual modelling to study the evolution of lizard coloration: sexual selection drives the evolution of sexual dichromatism in lacertids. Journal of Evolutionary Biology 26:1826-1835.

Pont AC, Meier R, 2002. The Sepsidae (Diptera) of Europe [electronic resource]: Brill.

Reichle D, 1968. Relation of Body Size to Food Intake, Oxygen Consumption, and Trace Element Metabolism in Forest Floor Arthropods. Ecology 49:538-542.

Ruxton GD, Sherratt TN, Speed MP, 2004. Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals and Mimicry. Oxford: Oxford University Press.

Schmid-Hempel P, 2003. Variation in immune defence as a question of evolutionary ecology. Proceedings of the Royal Society of London B: Biological Sciences 270:357-366.

Shoval O, Sheftel H, Shinar G, Hart Y, Ramote O, Mayo A, Dekel E, Kavanagh K, Alon U, 2012. Evolutionary Trade-Offs, Pareto Optimality, and the Geometry of Phenotype Space. Science 336:1157-1160.

Stearns SC, 1989. Trade-offs in life-history evolution. Functional ecology 3:259-268.

Svanbäck R, Eklöv P, 2011. Catch me if you can—predation affects divergence in a polyphenic species. Evolution 65:3515-3526.

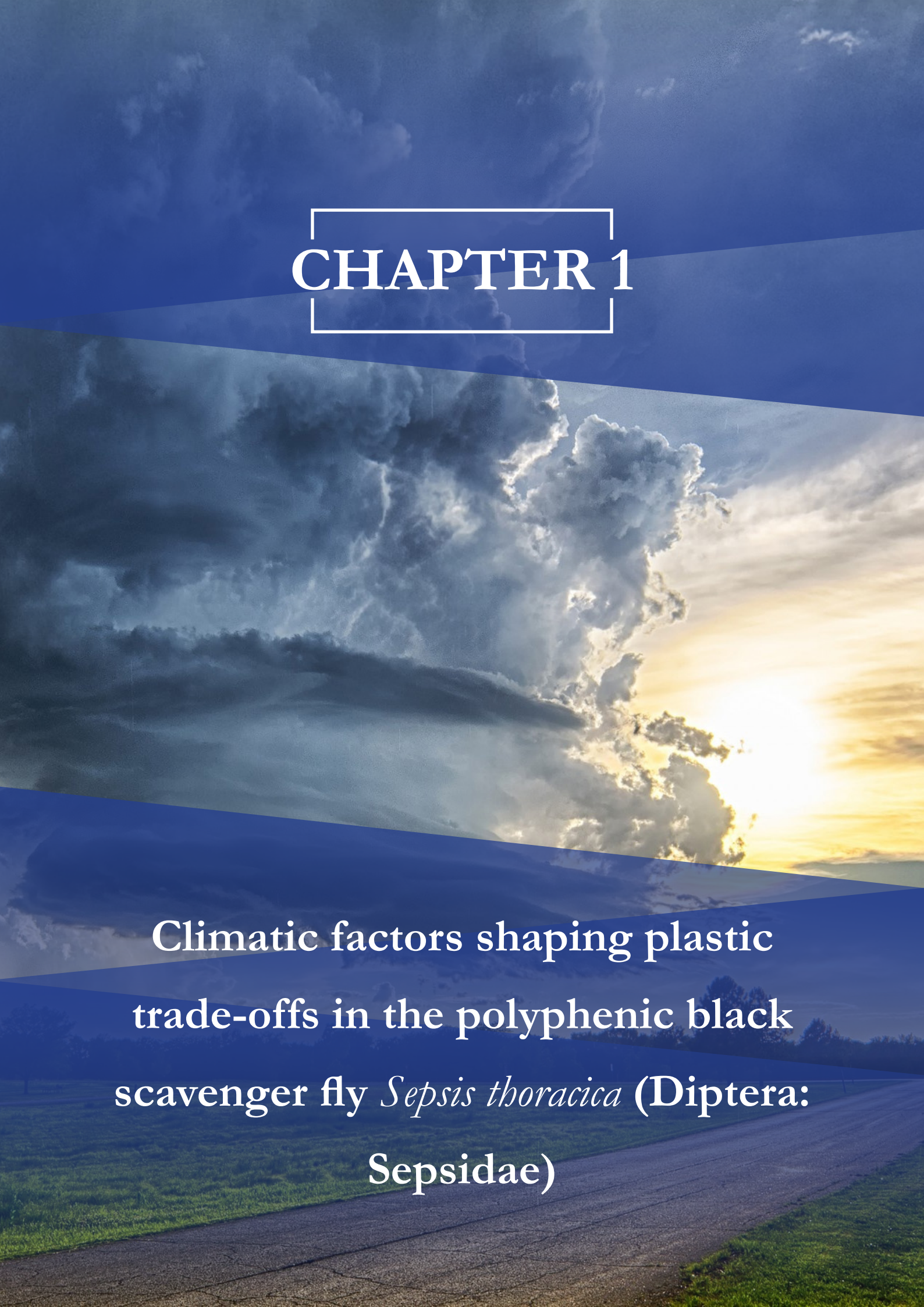
Talloen W, Dyck HV, Lens L, 2004. The cost of melanization: Butterfly wing coloration underenvironmental stress. Evolution 58:360-366.

True JR, 2003. Insect melanism: the molecules matter. Trends in ecology & evolution 18:640-647.

Whitman DW, Vincent S, 2008. Large size as an antipredator defense in an insect. Journal of Orthoptera Research 17:353-371.

Wooster D, 1994. Predator impacts on stream benthic prey. Oecologia 99:7-15.

Zera AJ, Harshman LG, 2001. The physiology of life history trade-offs in animals. Annual review of Ecology and Systematics:95-126.



CHAPTER 1

Climatic factors shaping plastic trade-offs in the polyphenic black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae)

CHAPTER 1

Manuscript Status: Submitted to Journal of Biogeography
Status in Journal: Major revisions

Climatic factors shaping plastic trade-offs in the polyphenic black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae)

Juan Pablo Busso [a]

[a] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zürich

ABSTRACT

Aim

Trade-offs allow individuals to optimize their fitness by tailoring the investment into different traits to various environmental conditions, such as over a latitudinal gradient. Along this gradient, trade-offs help adjust to thermal and insolation profiles, especially in small ectotherms, whose body temperature follows environmental temperatures closely. Two traits normally involved in latitudinal adaption are body size and melanism. Since both traits are very costly, individuals need to optimize the investment balance into each trait. In this study we addressed how environmental factors influence this trade-off in the short and long term.

Location

Europe

Methods

We raised flies from 15 latitudinal populations at three temperatures in a common garden experiment to differentiate plastic and evolutionary responses to temperature. We further analyzed how the different insolation components of the populations' habitats influenced the evolution of the trade-off.

Results

Male *Sepsis thoracica* feature a sigmoid trade-off between melanism and body size, defining two strikingly different male morphs: obsidian (small and black) and amber (large and yellow). The sigmoid trade-off relationship was altered by the developmental temperature, evincing its plasticity. The relationship further evolved across populations in response to the environmental characteristics of their habitat, notably temperature, insolation and UV radiation, suggesting that plasticity also has an underlying genetic basis. In contrast, latitude only influenced the extent of melanism but not body size.

Main Conclusions

As the plastic and evolutionary responses of the trade-off to temperature differed from one another, we can conclude that plasticity does not necessarily follow the direction of evolution of a trade-off, but rather adds to it. To our knowledge, this is the first study addressing the influence of environmental factors on plastic trade-offs, evincing their possible role in the evolution of polymorphisms.

KEYWORDS

body size, insolation, long-wave radiation, melanism, plasticity, short-wave radiation, temperature

INTRODUCTION

Trade-offs allow individuals to optimize their fitness in different environments by adjusting the relative investment into various traits to the environmental conditions. This differential investment allows species to extend their ranges, for example over a wide latitudinal gradient (Fleming & Gross, 1990; Ardia, 2005; Schiesari *et al.*, 2006; Laurila *et al.*, 2008). Along the latitudinal gradient individuals have to adapt to different season lengths, which involves adapting to different thermal profiles and also different insolation regimes. This adaptation can be achieved by adjusting trade-offs in order to optimize the phenotype to the particular set of environmental conditions of their habitat (Abraham & Jain, 2005; Shoval *et al.*, 2012). Such adaptation to environmental factors is of major importance for ectothermic organisms, such as insects, whose body temperature tends to follow environmental temperatures closely (Buckley *et al.*, 2012). Temperature predictably affects ectotherm body size, since a temperature increase typically results in a body size decrease (temperature-size rule: Atkinson, 1994; Klok & Harrison, 2013), providing one way by which body size is adjusted to different environmental thermal profiles. Though this decrease in size is entirely plastic, body size divergence among populations from different latitudes is often genetic (Partridge & Coyne, 1997; Chown & Gaston, 2010). Furthermore, body size is additionally affected by season length variation across latitudes, which can impose a time constraint to the individual's growth rate (Blanckenhorn & Demont, 2004; Nygren *et al.*, 2008). Hence, latitude can influence the ectotherm body size through temperature and/or season length.

Besides temperature and season length, insolation also changes drastically with latitude. The total daily solar energy decreases with latitude, and parallel to this insolation decrease many insects show increasing melanism (reviewed by Clusella Trullas *et al.*, 2007). Darker pigmentation facilitates absorption of the extra sunlight and helps increase the insect's body temperature (Watt, 1969; Wasserthal, 1975; Lindstedt *et al.*, 2009). Thus darker individuals can attain higher fitness at cooler conditions by being more active at suboptimal temperatures (Ellers & Boggs, 2002; Clusella Trullas *et al.*, 2007). As with body size, pigmentation differentiation among populations of different latitudes can be genetic (David *et al.*, 1985).

The aforementioned predicts that insects should show increased body size and melanism with latitude. However, both body size (Reichle, 1968) and pigmentation are resource-demanding traits, as melanin is costly to synthesize (Talloen *et al.*, 2004) and its synthesis further produces cytotoxic by-products (Schmid-Hempel, 2003; González-Santoyo & Córdoba-Aguilar, 2012). An organism with a widespread latitudinal distribution therefore will need to balance the allocation of resources between body size and melanism in optimizing its phenotype to the particular thermal and insolation conditions at each location.

An excellent system to address how temperature and insolation influence the trade-off between body size and pigmentation is *Sepsis thoracica* (Robineau-Desvoidy, 1830), which is the only European *Sepsis* that presents an orange coloration, while all its European close relatives are black (Pont & Meier, 2002). Males of this species exhibit extreme variability in size and color, with smaller individuals being mostly black, and large individuals being bright orange (Pont & Meier, 2002). This suggests a trade-off between pigmentation and body size. Because of its wide European range, *S. thoracica* allows the comparison of different populations across a wide latitudinal gradient and provides a unique opportunity to analyze how the environment influences the relative resource allocation to two costly phenotypic traits, here body size and melanism.

Since *S. thoracica* is a small species with short development time, we hypothesized that it would follow the temperature-size rule, as do other insects with similar life histories (Blanckenhorn & Demont, 2004; Berger *et al.*, 2013). We predicted that the temperature decrease with latitude would produce an increase in body size, and consequently an increase in orangeness. Alternatively, if insolation is more important, we hypothesized that a decrease in insolation with latitude will increase melanization, consequently decreasing body size as well. We raised flies from 15 latitudinal populations at three temperatures in a common garden experiment to differentiate plastic and evolutionary responses to temperature. By further considering the climatic conditions of the populations' habitat from data banks, we further attempted to uncover how the environment influences the evolution of the trade-off between body size and melanism.

MATERIALS AND METHODS

Population sampling and fly culture maintenance

We sampled 15 European *S. thoracica* populations along a latitudinal gradient: Pehka, Estonia (59.48° N, 26.37° W), Aarna, Estonia (58.07° N, 26.97° W), Ludwigshafen, Germany (49.48° N, 8.42° W), Nordrach, Germany (49.4° N, 8.08° W), Zürich, Switzerland (47.34° N, 8.54° W), Capriasca, Switzerland (46.07° N, 8.97° W), Borgonuovo, Italy (46.33° N, 9.44° W),

Asturias, Spain (43.3° N, 6.0° E), Petroia, Italy (43.23° N, 12.56° W), Terni, Italy (42.57° N, 12.62° W), Prati di Stronconi, Italy (42.48° N, 12.68° W), Padula, Italy (40.34° N, 15.66° W), 30 km east of Padula, Italy (40.34° N, 15.66° W), Lamezzia, Italy (38.92° N, 16.25° W), and Adrano, Italy (37.67° N, 14.83° W). Wild-caught females were brought to the laboratory and used to establish cultures of multiple (5–25) replicate iso-female lines per population, which were housed in separate plastic containers and regularly supplied with fresh cow dung, sugar, and water ad libitum. The cultures were maintained at 18 ± 1 °C under a 14:10 h light:dark cycle.

Rearing latitudinal lines

To analyze how environmental factors shape the trade-off between body size and melanism, we reared multiple lines (2-10) from the various populations in a common garden. A small container with more or less dung, to generate individuals of varying sizes through larval food competition, was placed for 24 hours inside any of the *S. thoracica* stock cultures for females to deposit eggs. This dung was incubated afterwards in a novel container in climate chambers at 60% humidity, and 14:10 h light:dark cycle to obtain adult flies. Flies were reared at three different temperatures: 18 °C, 24 °C, and 30 °C. This method of using iso-female lines instead of wild-caught females permits separation of genetic, plastic and environmental influences on body size and melanism. Since sepsid larvae and pupae develop within the dung pats into which the females laid their eggs, insolation will not reach them directly, but it will influence the temperature of the dung pat. Because of this indirect influence through temperature, we decided to focus on the effects of temperature during development.

Morphometric measurements

Emerging individuals were dissected and their foreleg femur was photographed under a stereo microscope MZ12 with a DFC490 camera (Leica), against a neutral white background. The camera was calibrated with a mini IT-8 calibration target, in order to guarantee color consistency between the different pictures. The target also served as a scale to posteriorly measure size. We used a self-written code in ImageJ, which measured the amount of pixels of the foreleg femur, and converted them to the real area according to the scale correspondent to each picture. The code also measured the amount of melanic pixels in the femur, guaranteeing an objective quantification of melanism. The melanic color of the flies was defined as any pixel with a V value over 163, in the YUV colorspace. This threshold was selected based on the valley of the bimodal distribution of the V values present in the femur coloration. Melanism represented the proportion of black and brown pixels over all the pixels of the femur.

Environmental variables

The thermal and insolation profiles of the various locations were obtained from the ECMWF's ERA-Interim database (Dee *et al.*, 2011). We retrieved all the main variables related to temperature and insolation available in the dataset. For each location we obtained the mean temperature, and insolation was decomposed into its different components: surface solar radiation downwards (short-wave direct and diffuse radiation that reaches the Earth's surface), surface net solar radiation (net short-wave radiation after subtracting the part reflected by the earth), surface thermal radiation downwards (long-wave radiation reaching the Earth's surface), and downward UV radiation at the surface (UV radiation reaching the Earth's surface). We then averaged the values between 1979 and 2016 for the warmest months (May to October) and used these averages as predictor variables in the models described below. We also considered latitude and the length of the season as the number of days in which the mean temperature remained above 9 °C (cf. Blanckenhorn, 1999).

Statistical analyses

The males' multimodal distribution in melanism with 2 valleys was used to determine threshold values for the separation of the two male morphs: obsidians (black and small) and amber (orange and large). Situated between these morphs we found some intermediate phenotypes as well. The male morphs plus the females composed the levels of the factor *morph* in the following model.

To determine if latitude influenced body size and melanism we fitted two models, one for body size and one for melanism. For body size we fitted a linear mixed effect model with latitude, developmental temperature, and morph plus all two-way interactions between them (N = 4016). For melanism, a proportional response variable, we used an analogous generalized mixed effect model fitting a logistic regression to the data. Since the females are all black, we used only males for this model (N = 2998), so only latitude, developmental temperature and the interaction between them was entered. We also incorporated in all models a random effect of line nested within population to control for genetic relatedness. We used the package 'lme4' to test the significance of each variable in each model.

To first analyze the general relationship between body size and melanism in *S. thoracica* males we fitted a 5-Parameter logistic regression model: *Melanism* = *c* + (*d*-*c*)/[1+(BS/*e*)^*b*]^*f* , where BS is body size, *d* is the estimated melanism at body size zero, *c* is the estimated melanism at infinite body size, *e* is the mid-range body size, *b* is the slope factor, and *f* is the asymmetry factor (Gottschalk & Dunn, 2005). This regression was independently fitted to each line of each population at every developmental temperature, to determine if the sigmoid curves differ significantly between populations. We studied a total of 98 iso-female lines, which rendered it difficult to achieve a fully factorial design (i.e. all lines at all temperatures) due to some lines dying in the process. Nevertheless, all populations were represented by at least 3 lines at all temperatures. We further calculated the derivative of each curve to obtain the maximum slope at the inflection point, yielding the body size at which the curve changes from concave to convex. (It is worth noting that, mathematically, the maximum slope is not the same as the slope factor, and the inflection point is not the same as the mid-range body size.) From each population fit we calculated the residuals, which were then squared an added to obtain a sum of squares that represented the overall error (SS_{error}). We similarly added the degrees of freedom of all the population models to obtain the degrees of freedom of the error (df_{error}). Then we fitted a 5-Parameter logistic regression model to the entire male dataset (N = 2998) to evaluate the general model. We squared and added the residuals of the general model to obtain the total error (SS_{total}), and calculated an F-statistic with the following formula:

$$F = \frac{(SS_{total} - SS_{error})/(df_{total} - df_{error})}{(SS_{error}/df_{error})}$$

The F-value and the degrees of freedom were used to calculate the p-value of the population differences. These calculations were necessary because a regular ANOVA does not allow fitting a 5-parameter logistic regression to the data.

To then analyze the effects of temperature and insolation on the body size – melanism trade-off, we fitted a model to each of the 5 parameters of the logistic regression as the outcome variable. Each model included developmental temperature and all above-mentioned environmental variables, which are population characteristics. Interactions between developmental temperature and the environmental variables were first entered but later removed from the models because they were always non-significant. We used the package 'lme4' to test the significance of each variable in each model. Since the model only investigates 15 populations and the environmental variables are population-specific, this does not leave a lot of power a priori to analyze interactions between all the environmental variables. We again also incorporated in the models line nested within population as random effects. All analyses were done using the software R Version 3.2.2 (R Core R Development Core Team, 2015).

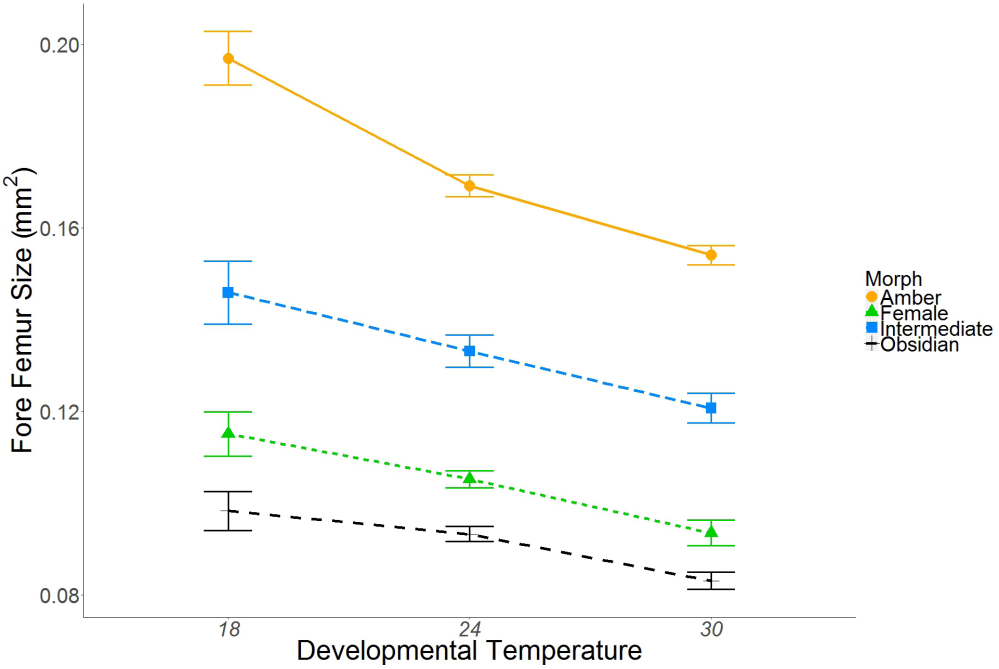


Figure 1. Differences in body size between females and male morphs at different development temperature.

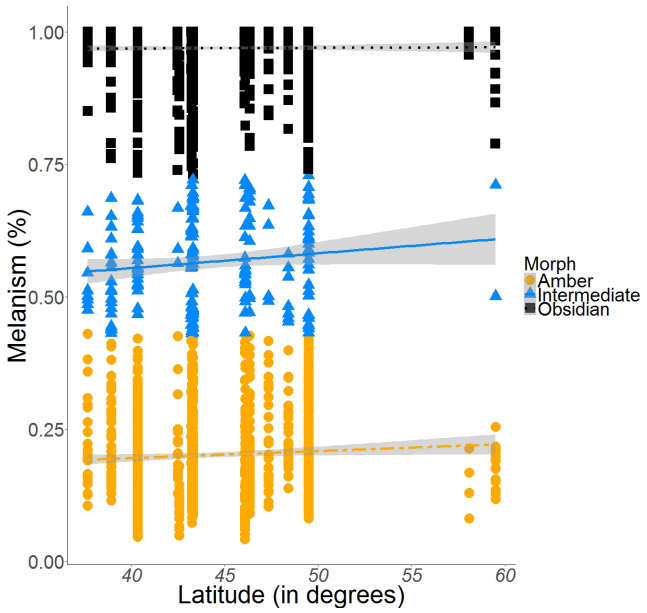


Figure 2. Influence of latitude on melanism.

RESULTS

Effect of rearing temperature and latitude on body size and melanism

Body size was negatively affected by developmental temperature in accordance with the temperature-size-rule (Fig. 1, estimate = -1.007 e^{-2} ± 0.962 e^{-2}, X^2=235.56, df = 1, p < 0.001) and differed significantly between the morphs (Fig. 1, X^2= 6676.59, df = 3, p<0.001). The interaction between temperature and morph was also significant (Fig. 1, X^2= 66.75, df = 3, p < 0.001). Latitude showed no significant main or interactive effects on body size (*latitude*, X^2= 0.257, df=1, p=0.612; *latitude:temperature*, X^2= 0.624, df = 1, p = 0.430; *latitude:morph*, X^2= 3.108, df = 3, p = 0.375). By contrast, melanism increased with latitude (Fig. 2, estimate = 0.105± 0.095, X^2= 6.329, df=1, p = 0.012), with developmental temperature and the interaction between the two variables showing no significant effect (*temperature*, X^2=0.351, df = 1, p=0.554; *latitude:temperature*, X^2= 0.017, df = 1, p = 0.898).

Body size – melanism trade-off

We found a sigmoid relationship between body size and melanism in *S. thoracica,* which was present in all 15 sampled populations, although the curves differed significantly between populations ($F_{85,2835}$=6.566, *P*<0.001). While the body size distribution was unimodal, melanism showed a bi- to slightly trimodal distribution (Fig. 3), which we used post-hoc to determine the morphs of the species. As there were actually two valleys with a slight additional mode

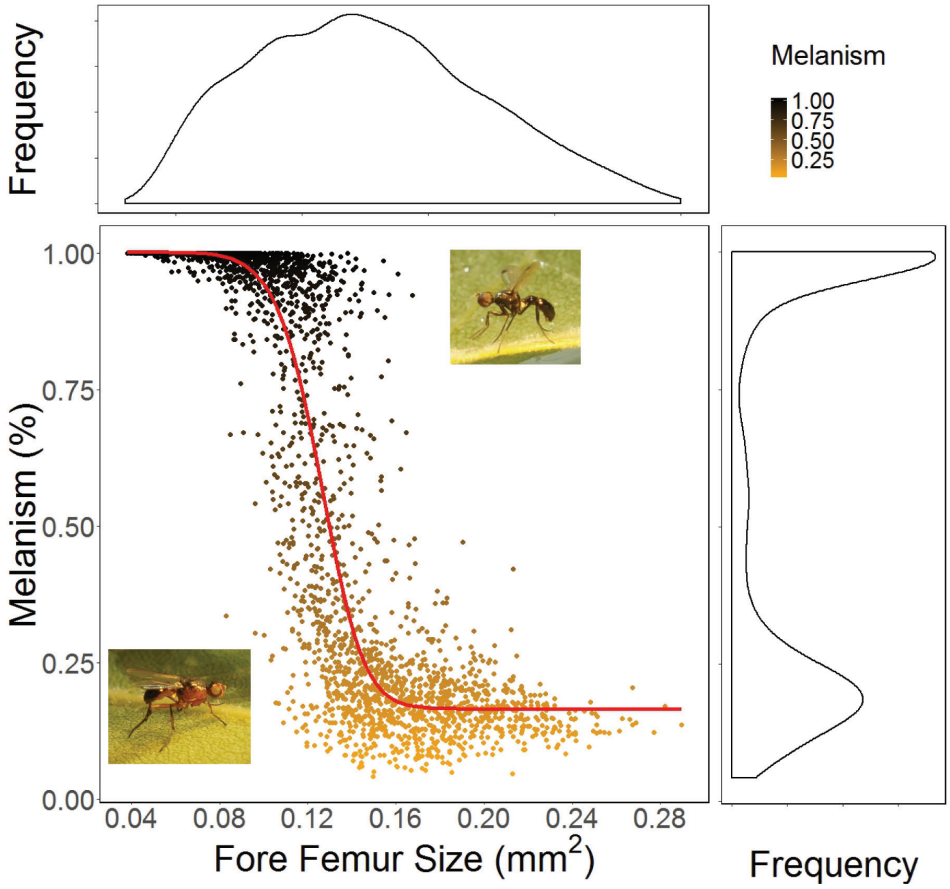


Figure 3. Sigmoid relationship between body size and melanization in *Sepsis thoracica*, with their respective frequency distributions for males. The pictures show the amber and obsidian male morphs.

Table 1. Influence of environmental variables on the parameters of the 5 Parameter Logistic regression.

	Parameters						
Environmental Variables	**b**	**c**	**d**	**e**	**f**	**Inflection Point**	**Minimum Slope**
Developmental Temperature	-8821.633 (± 73537.844)	-8.075 (± 12.558)	23.252 (± 14.954)	-0.003 (± 0.001)***	0.97 (± 0.031)	-0.002 (± 0.001)***	-1.009 (± -0.021)
Surface Solar Radiation Downwards	-3.833 (± 1.453)**	-0.02 (± 0.017)	0.119 (± 0.063)	-0.192 (± 0.061)*	0.013 (± 0.034)	-0.09 (± 0.069)	-17.962 (± -27.718)
Surface Net Solar Radiation	-41.659 (± 148.636)	0.024 (± 0.02)	-1.286 (± 4.352)	0.041 (± 0.054)	1.757 (± 4.204)	0.007 (± 0.041)	-0.366 (± -0.508)
Downward UV radiation at the surface	3.509 (± 1.702)*	0.124 (± 0.91)	-0.127 (± 0.096)	0.152 (± 0.086)	32.918 (± 125.224)	0.078 (± 0.09)	-0.156 (± -0.343)
Surface thermal radiation downwards	-81.772 (± 85.54)	-0.072 (± 0.029)*	-3.547 (± 4.813)	-0.013 (± 0.008)	0.606 (± 0.21)	-0.011 (± 0.006)	-1.729 (± -0.345)**
Environmental Surface Temperature	46.942 (± 35.165)	0.07 (± 0.033)*	-0.479 (± 0.295)	0.014 (± 0.01)	2.145 (± 0.946)	0.013 (± 0.02)	-0.65 (± -0.17)
Season Length	-304.587 (± 3687.266)	0.06 (± 0.061)	0.208 (± 0.227)	0.017 (± 0.025)	1.187 (± 1.287)	0.001 (± 0.017)	-0.705 (± 0.447)

The numbers represent changes in one Standard Deviation due to an increase of one unit in the environmental variable. * P < 0.05; ** P< 0.01; *** P< 0.001. Refer to Figure 4 for a visual representation of these values.

in the middle, this yielded the following threshold values for the separation of the males into morphs: the first valley in melanism at 0.73 marked the lower limit of the Obsidian morph, and the second valley at 0.43 marked the upper limit of the Amber morph. The remaining males were classified as intermediate (Fig.3).

Effect of developmental temperature and environmental variables on the trade-off

Table 1 lists the relative effects of each environmental variable on the 5 parameters of the logistic regression.To illustrate the complex numerical interpretation of these parameters, Fig. 4 depicts the changes in the mean sigmoid curves (based on the entire dataset) resulting from an increase by one standard deviation (SD) in only the significant regression parameters of all tested environmental variables. Figure 5 depicts the curve differences between the two most extreme latitudinal populations.

DISCUSSION

Males display a sigmoid relationship between body size and melanism, resulting in a body size threshold that divides males into two color morphs: obsidian (small and black) and amber (large and yellow). Their body sizes differ significantly from one another as well as from the females, which are of intermediate size (Fig. 1). Body size was also negatively influenced by the developmental temperature according to the temperature-size rule (Atkinson, 1994). On the other hand, melanism of *S. thoracica* males increased with latitude, as expected (see Introduction), whereas body size did not vary with latitude, contrary to expectation. We also found that the sigmoid curve itself was differently affected by developmental temperature and some of the environmental habitat characteristics considered (Fig. 4a-e). We can see that the plastic response of the curve to developmental temperature differs from its evolutionary response to the environmental surface temperature (Fig. 4a vs. 4b). Moreover, this sigmoid relationship also showed various evolutionary responses to the different types of radiation composing insolation (4b,d,e). Hence, temperature and insolation appear to have played complementary roles in shaping the trade-off relationship in the various latitudinal populations.

In *S. thoracica* males, body size and melanism

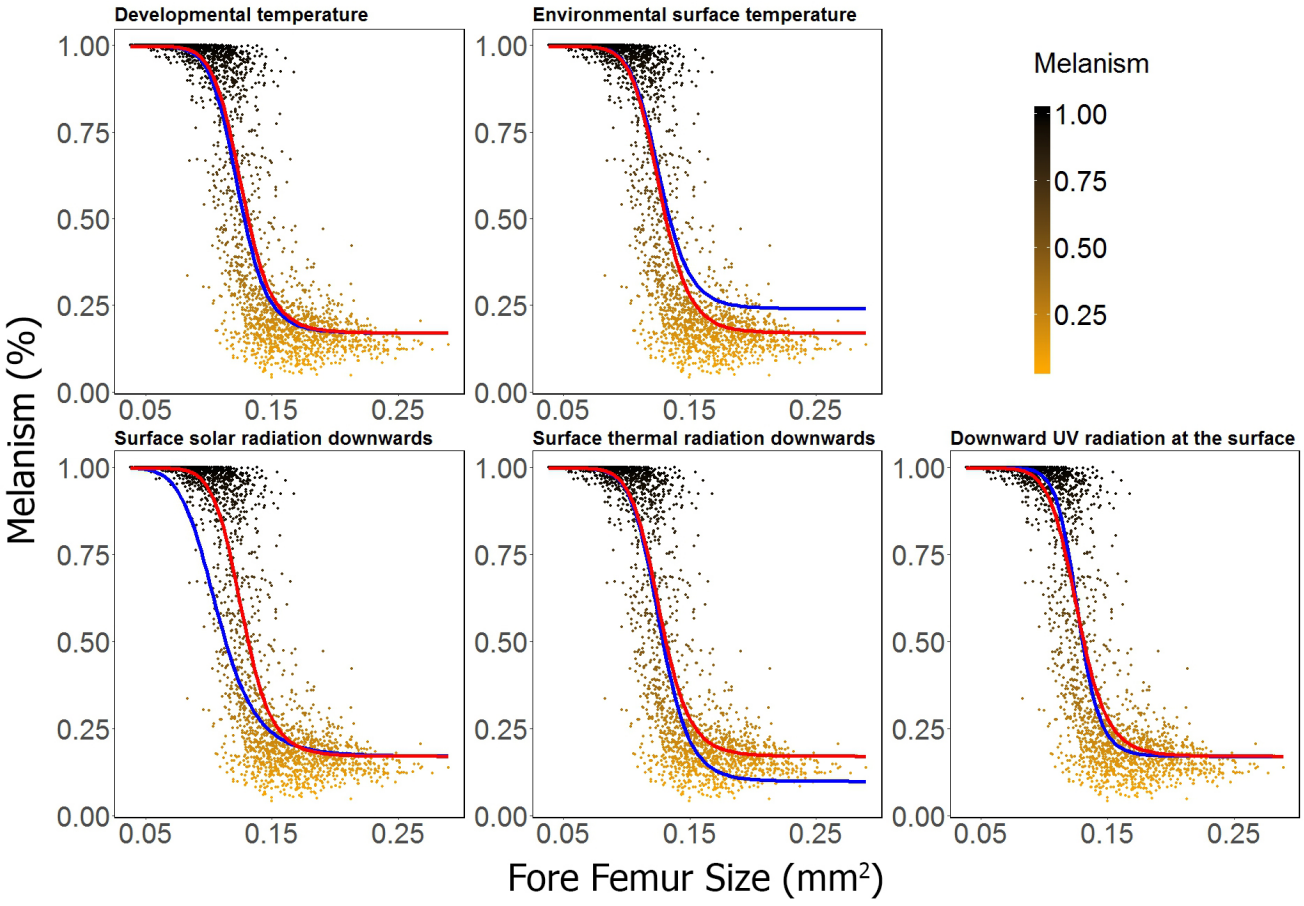


Figure 4. Effect of various environmental factors on the sigmoid trade-off between body size and melanism. The blue curve represents the changes on the mean sigmoid curve (red curve) resulting from an increase in one Standard Deviation of only the regression parameters significantly influenced by the environmental variable.

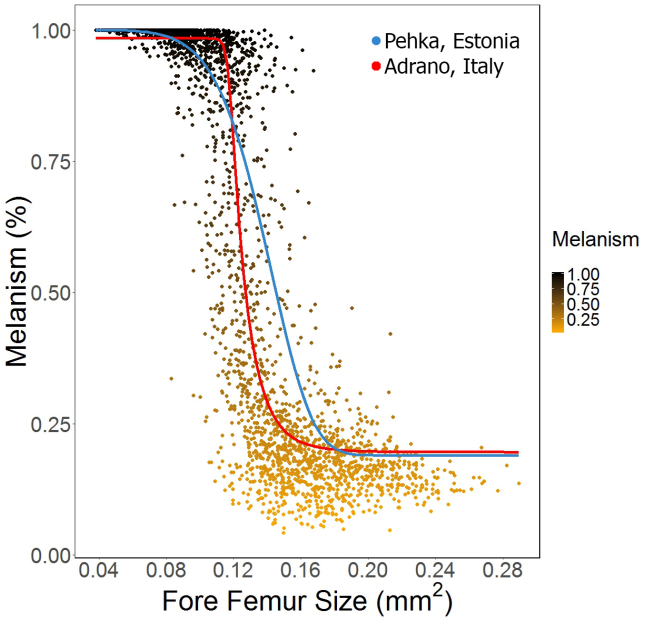


Figure 5. Actual differences in the sigmoid trade-off between the northernmost (Pehka, Estonia) and the southernmost *S. thoracica* population (Adrano, Italy) sampled.

present a sigmoid relationship, which evinces a complex trade-off involving a body size threshold above which the individuals dramatically decrease their investment in melanin, becoming almost completely amber (Fig. 3). Such threshold traits are commonly related to polymorphisms (Taborsky & Brockmann, 2010), as observed e.g. in the horned beetle *Ontophagus acuminatus* (Emlen, 1997) and other dung beetles (Moczek *et al.*, 2002; Macagno *et al.*, 2011). In *S. thoracica* the steep slope of the sigmoid curve divided the males into two morphs: obsidian (small and black) and amber (large and orange). Since we found both male morphs in all isofemale lines of all populations, we conclude that the morphs are not determined genetically but rather by condition-dependent plasticity. Nevertheless, the sigmoid relation differed between populations, even when the environment was held constant, which suggests that the threshold curve itself has a genetic basis (Taborsky & Brockmann, 2010), allowing the trade-off to be shaped by selection.

The sigmoid trade-off relationship exhibited a short-term plastic response to developmental temperature and also long-term population differentiation, presumably an evolutionary response due to local adaptation, to the environmental surface temperature, but these two responses to temperature differed from one another. While an increase in developmental temperature shifted the curve slightly leftwards (Fig. 4a), increased environmental surface temperature primarily strongly augmented melanization of the amber morph (Fig. 4b). Developmental temperature is expected to shift the curve leftwards by reducing the body size of the flies according to the temperature-size-rule (Atkinson, 1994; Fig. 1), as also observed in another sepsid (Berger *et al.*, 2013). The generality of this rule, which is widespread among arthropods (Klok & Harrison, 2013), results from temperature differently influencing growth and developmental rates (Forster *et al.*, 2011; Forster & Hirst, 2012; Zuo *et al.*, 2012). Alternatively, the shift in the curve could be due to developmental temperature influencing the melanization rate (Gibert *et al.*, 1996; Solensky & Larkin, 2003), reducing the melanin production in a certain body size range of *S. thoracica* males. Either mechanism, or both combined, results in a leftward shift of the curve with increasing temperature, thus reducing melanism in flies of intermediate body sizes. Such reduction in melanism decreases the absorption of radiation (Mason, 1948; Hackman, 1953; Murkin & Arango, 2009), causing lower body temperatures (Watt, 1969; Wasserthal, 1975; Lindstedt *et al.*, 2009). By this strategy individuals could avoid overheating. Nevertheless, an organism's body temperature depends not only on its melanism, but also on its body size (Digby, 1955; Willmer & Unwin, 1981). Hence, the reduction of melanism at particular body sizes suggests a delicate balance between the two traits to influence body temperature.

On the other hand, when considering the environmental characteristics of the populations, an increase in environmental surface temperature results in an increase of melanism in amber males (Fig. 4b). This increase is counterintuitive because it would raise the insect's body temperature (Watt, 1969; Wasserthal, 1975; Lindstedt *et al.*, 2009), potentially leading to overheating. However, this effect could be countered by the surface thermal radiation downwards (Fig. 4d). Thermal radiation represents the long-wave radiation emitted by Earth and its atmosphere and plays an important role for *S. thoracica*, because the dung pats where the flies feed and reproduce absorb a large part of short-wave solar radiation due to their dark coloration, and emit long-wave radiation in return. Long-wave radiation absorbance is not influenced by melanism because it exceeds the pigment's absorbance spectrum (Mason, 1948; Hackman, 1953; Murkin & Arango, 2009), but it is affected by body size (Digby, 1955; Willmer & Unwin, 1981). Larger individuals have lower heat exchange rates due to their lower surface-to-volume ratio, and they reach a higher temperature excess, which is the difference between the body temperature and the ambient air temperature (Digby, 1955; Willmer & Unwin, 1981). Consequently, larger amber individuals are more prone to overheating, and they may compensate this by lower melanism, reducing the absorption of short-wave radiation. This decrease in melanism in the larger (amber) males responding to the increase in long-wave radiation (Fig.

4d) would cancel out the increase in melanism due to the environmental surface temperature (upward shift) seen in Fig. 4b. These opposing effects could suggest the evolution of an equilibrated response to maintain low melanism in the amber males, which may have other advantages (e.g. in sexual selection: Busso et al. in prep).

Melanin absorbs short-wave radiation (Mason, 1948; Hackman, 1953; Murkin & Arango, 2009), and this radiation (surface solar radiation downwards) shifts the curve leftwards and reduces the steepness of its slope (Fig. 4c). The absorption of short-wave radiation results in increased body temperature (Watt, 1969; Wasserthal, 1975; Lindstedt *et al.*, 2009), thus raising overheating risk. Furthermore, melanin content also increases the rate of heat exchange of the individual (Brakefield & Willmer, 1985). Hence, the lower the melanism of an individual, the less it will have to be reduced to avoid overheating. The low melanism of the amber males probably already results in low to null absorbance of short-wave radiation; therefore, no further reduction in melanism is needed to avoid overheating. The small obsidians, in contrast, probably need to maintain high melanism to compensate for their high rates of heat loss due to their greater surface-to-volume ratio (Digby, 1955; Willmer & Unwin, 1981). As a result, although melanin absorbs short-wave radiation, absorbance crucially depends on body size, resulting in complex effects of short-wave radiation over the body size range of the flies.

Another insolation component that influenced the curve was UV radiation (Fig. 4e). UV radiation has lethal effects on insects (Beard, 1972; Faruki *et al.*, 2007; Ghanem & Shamma, 2007), as it directly damages DNA (Pfeifer, 1997) and/or enhances the production of reactive oxygen species (Meng *et al.*, 2009; Sang *et al.*, 2012). However, melanin protects cells against these harmful effects of UV radiation (Ortonne, 2002; Kvam & Dahle, 2003; Tada *et al.*, 2010), and this defensive role is also important in insects (Mosse & Lyakh, 1994; Hu *et al.*, 2013; Bastide *et al.*, 2014; Debecker *et al.*, 2015), but see Gunn (1998). Consequently we would expect an increase in melanism with an increase in UV radiation for all individuals, but instead we observed an increase in the steepness of the slope (Fig. 4e). This change in slope reinforces the subdivision of males into two morphs by curtailing the body size range for intermediate phenotypes, again suggesting that the morphs indeed could be using two different protection strategies against UV radiation. Obsidians invest in melanin, which protects the individuals against UV radiation by directly absorbing it (Mason, 1948; Hackman, 1953; Murkin & Arango, 2009), whereas ambers alternatively invest more into body size. Since the thickness of insects' cuticle increases with body size (Evans & Sanson, 2005), and UV radiation has a very low penetration (Meinhardt *et al.*, 2008), it is possible that beyond a threshold body size (inflection point of the curve), the insect's cuticle is thick enough to stop the penetration of UV radiation altogether. Consequently, melanin would no longer be needed above this threshold and resources could be redirected into body size. This would result in a melanin - body size trade off, as also present in some other insects (Cotter *et al.*, 2008; Harris *et al.*, 2012). Alternatively, ambers could invest in a suite of protective macromolecules (heat shock proteins, cytochrome P450, and antioxidant enzymes) as safeguards against UV radiation (Meng *et al.*, 2009; Meng *et al.*, 2010; Feyereisen, 2011). However, the latter scenario is less likely, since to our knowledge there is no evidence for a trade-off between melanin and these protective macromolecules in the literature. Future work should address in detail the effect of UV radiation on the production of melanin as well as the role of protective macromolecules in both morphs to disentangle their effects. An increase in UV radiation implies greater risk of damage, which would reduce the fitness of an inefficient intermediate strategy. Consequently, the steeper slope in response to an increase in UV radiation would reduce intermediate phenotypes and assure that the males are protected either by being fully melanic or by a thick enough cuticle.

All these environmental factors do not act separately, but rather simultaneously on this sigmoid trade-off. Figure 5 shows the actual integrative effects of the different factors as illustrated by the contrast between the northernmost (Pehka, Estonia) and the southernmost *S. thoracica* population (Adrano, Italy). The effects of environmental surface temperature and surface thermal radiation downwards (long-wave radiation; cf. Fig. 4b,d) on the melanism of the amber males appear to cancel each other out, resulting in no melanism change. The increase in surface solar radiation downwards (short-wave), however, shifts the southern population leftwards, while the increase in downward UV radiation at the surface increases the steepness of its slope. These effects result in an overall increase in melanism with latitude, which concurs with what has been observed in other insects (reviewed by Clusella Trullas *et al.*, 2007), and which we also found here across our 15 populations (Fig. 2). Season length did not significantly influence the trade-off curve. The short development time of *S. thoracica* (Pont & Meier, 2002) allows for multivoltinism even in the northernmost populations, which dilutes and ultimately probably removes strong effects of seasonal time constraints on this species' life history

(Blanckenhorn & Demont, 2004; Nygren *et al.*, 2008). Hence, the integrative influence of temperature and insolation on the curve seems to mostly affect the intermediate states of the trade-off, leaving the extreme morphs largely unaltered.

Both morphs were present in all isofemale lines, indicating that the morphs are not genetically determined, but rather represent a plastic polyphenism. We showed that the trade-off between body size and melanism not only represents plasticity, but is also plastic itself. Developmental temperature slightly reshapes the trade-off, adjusting the male morphospace to the different thermal profiles. Hence, this plastic response can act as a buffer when facing new environmental conditions resulting, for example, from climate change (Chown *et al.*, 2007; Boggs, 2016; Sgrò *et al.*, 2016). At the same time the sigmoid trade-off differed significantly among populations even when the environment was held constant, demonstrating its genetic basis (Taborsky & Brockmann, 2010). However, the plastic response to temperature differed from the evolutionary response (Fig. 4a,b), highlighting that plasticity does not necessarily need to match the direction of the evolution of the trade-off (Fusco & Minelli, 2010; overview by Minelli, 2015). Temperature and insolation appear to have played complementary roles in the trade-off evolution in the various populations. The correlative nature of our approach does not allow us to test the causation of the evolutionary changes; nevertheless, our results suggest roles of the various climatic factors considered for the evolution of the body size - melanism trade-off in *S. thoracica*.

To conclude, latitude influences the extent of melanism but not body size in *S. thoracica*, but other climatic variables further contribute to geographic differentiation among populations that can be interpreted as adaptive. The sigmoid trade-off between melanism and body size in male *S. thoracica* not only defines the plasticity of the male phenotypes, but is also plastic itself. It seems to evolve across populations in response to various climatic factors such as temperature, insolation and UV radiation, suggesting that plasticity has an underlying genetic basis. As the plastic and evolutionary responses to temperature (and other factors) differed from one another, plasticity does not necessarily follow the direction of evolution of the trade-off, but rather adds to it. The environmental effects on body size and melanism demonstrated here will further influence the fitness of *S. thoracica* individuals beyond their role in sexual selection (Partridge, 1987; Andersson, 1994; Houde, 1994; Puniamoorthy *et al.*, 2012a; Puniamoorthy *et al.*, 2012b) and predation vulnerability (Busso and Blanckenhorn, 2017; Berger *et al.*, 2006; Whitman & Vincent, 2008; Svanbäck & Eklöv, 2011). To our knowledge, this is the first study addressing the influence of climatic factors on plastic trade-offs, evincing their possible role of for the evolution of polymorphisms.

REFERENCES

Abraham, A. & Jain, L. (2005) Evolutionary multiobjective optimization. *Evolutionary Multiobjective Optimization*, pp. 1-6. Springer.

Andersson, B. (1994) *Sexual Selection*. Princeton University Press.

Ardia, D.R. (2005) Tree swallows trade off immune function and reproductive effort differently across their range. *Ecology*, **86**, 2040-2046.

Atkinson, D. (1994) *Temperature and Organism Size: A Biological Law for Ectotherms?* Academic Press.

Bastide, H., Yassin, A., Johanning, E.J. & Pool, J.E. (2014) Pigmentation in *Drosophila melanogaster* reaches its maximum in Ethiopia and correlates most strongly with ultra-violet radiation in sub-Saharan Africa. *BMC Evolutionary Biology*, **14**, 179.

Beard, R.L. (1972) Lethal Action of UV Irradiation on Insects. *Journal of Economic Entomology*, **65**, 650-654.

Berger, D., Walters, R. & Gotthard, K. (2006) What keeps insects small?—Size dependent predation on two species of butterfly larvae. *Evolutionary Ecology*, **20**, 575-589.

Berger, D., Postma, E., Blanckenhorn, W.U. & Walters, R.J. (2013) Quantitative genetic divergence and standing genetic (co)variance in thermal reaction norms along latitude. *Evolution*, **67**, 2385-99.

Blanckenhorn, W.U. (1999) Different growth responses to temperature and resource limitation in three fly species with similar life histories. *Evolutionary Ecology*, **13**, 395-409.

Blanckenhorn, W.U. & Demont, M. (2004) Bergmann and Converse Bergmann Latitudinal Clines in Arthropods: Two Ends of a Continuum? *Integrative and Comparative Biology*, **44**, 413-424.

Boggs, C.L. (2016) The fingerprints of global climate change on insect populations. *Current Opinion in Insect Science*, **17**, 69-73.

Brakefield, P.M. & Willmer, P.G. (1985) The basis of thermal melanism in the ladybird *Adalia bipunctata*: Differences in reflectance and thermal properties between the morphs. *Heredity*, **54**, 9-14.

Buckley, L.B., Hurlbert, A.H. & Jetz, W. (2012) Broad-scale ecological implications of ectothermy and endothermy in changing environments. *Global Ecology and Biogeography*, **21**, 873-885.

Chown, S.L. & Gaston, K.J. (2010) Body size variation in insects: a macroecological perspective. *Biological Reviews*, **85**, 139-169.

Chown, S.L., Slabber, S., McGeoch, M.A., Janion, C. & Leinaas, H.P. (2007) Phenotypic plasticity mediates climate change responses among invasive and indigenous arthropods. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 2531-2537.

Clusella Trullas, S., van Wyk, J.H. & Spotila, J.R. (2007) Thermal melanism in ectotherms. *Journal of Thermal Biology*, **32**, 235-245.

Cotter, S., Myatt, J., Benskin, C. & Wilson, K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology*, **21**, 1744-1754.

David, J., Capy, P., Payant, V. & Tsakas, S. (1985) Thoracic trident pigmentation in *Drosophila melanogaster*: differentiation of geographical populations. *Genet. Sel. Evol*, **17**, 211-224.

Debecker, S., Sommaruga, R., Maes, T. & Stoks, R. (2015) Larval UV exposure impairs adult immune function through a trade-off with larval investment in cuticular melanin. *Functional Ecology*, **29**, 1292-1299.

Dee, D.P., Uppala, S.M., Simmons, A.J., Berrisford, P., Poli, P., Kobayashi, S., Andrae, U., Balmaseda, M.A., Balsamo, G., Bauer, P., Bechtold, P., Beljaars, A.C.M., van de Berg, L., Bidlot, J., Bormann, N., Delsol, C., Dragani, R., Fuentes, M., Geer, A.J., Haimberger, L., Healy, S.B., Hersbach, H., Hólm, E.V., Isaksen, L., Kållberg, P., Köhler, M., Matricardi, M., McNally, A.P., Monge-Sanz, B.M., Morcrette, J.J., Park, B.K., Peubey, C., de Rosnay, P., Tavolato, C., Thépaut, J.N. & Vitart, F. (2011) The ERA-Interim reanalysis: configuration and performance of the data assimilation system. *Quarterly Journal of the Royal Meteorological Society*, **137**, 553-597.

Digby, P.S. (1955) Factors affecting the temperature excess of insects in sunshine. *Journal of Experimental Biology*, **32**, 279-298.

Ellers, J. & Boggs, C.L. (2002) The evolution of wing color in *Colias* butterflies: heritability, sex linkage, and population divergence. *Evolution*, **56**, 836-840.

Emlen, D.J. (1997) Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Behavioral Ecology and Sociobiology*, **41**, 335-341.

Evans, A.R. & Sanson, G.D. (2005) Biomechanical properties of insects in relation to insectivory: cuticle thickness as an indicator of insect'hardness' and'intractability'. *Australian Journal of Zoology*, **53**, 9-19.

Faruki, S., Das, D., Khan, A. & Khatun, M. (2007) Effects of ultraviolet (254nm) irradiation on egg hatching and adult emergence of the flour beetles, *Tribolium castaneum, T. confusum* and the almond moth, *Cadra cautella*. *Journal of Insect Science*, **7**, 36.

Feyereisen, R. (2011) Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, **1814**, 19-28.

Fleming, I.A. & Gross, M.R. (1990) Latitudinal clines: a trade-off between egg number and size in Pacific salmon. *Ecology*, 2-11.

Forster, J. & Hirst, A.G. (2012) The temperature-size rule emerges from ontogenetic differences between growth and development rates. *Functional Ecology*, **26**, 483-492.

Forster, J., Hirst, A.G. & Atkinson, D. (2011) How do organisms change size with changing temperature? The importance of reproductive method and ontogenetic timing. *Functional Ecology*, **25**, 1024-1031.

Fusco, G. & Minelli, A. (2010) Phenotypic plasticity in development and evolution: facts and concepts. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 547-556.

Ghanem, I. & Shamma, M. (2007) Effect of non-ionizing radiation (UVC) on the development of *Trogoderma granarium* Everts. *Journal of stored products research*, **43**, 362-366.

Gibert, P., Moreteau, B., Moreteau, J.-C. & David, J.R. (1996) Growth temperature and adult pigmentation in two *Drosophila* sibling species: an adaptive convergence of reaction norms in sympatric populations? *Evolution*, 2346-2353.

González-Santoyo, I. & Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata*, **142**, 1-16.

Gottschalk, P.G. & Dunn, J.R. (2005) The five-parameter logistic: A characterization and comparison with the four-parameter logistic. *Analytical Biochemistry*, **343**, 54-65.

Gunn, A. (1998) The determination of larval phase coloration in the African armyworm, *Spodoptera exempta* and its consequences for thermoregulation and protection from UV light. *Entomologia Experimentalis et Applicata*, **86**, 125-133.

Hackman, R. (1953) Chemistry of insect cuticle. 3. Hardening and darkening of the cuticle. *Biochemical Journal*, **54**, 371.

Harris, R., McQuillan, P. & Hughes, L. (2012) Patterns in body size and melanism along a latitudinal cline in the wingless grasshopper, *Phaulacridium vittatum*. *Journal of biogeography*, **39**, 1450-1461.

Houde, A.E. (1994) Effect of artificial selection on male colour patterns on mating preference of female guppies. *Proceedings of the Royal Society of London B: Biological Sciences*, **256**, 125-130.

Hu, Y.G., Shen, Y.H., Zhang, Z. & Shi, G.Q. (2013) Melanin and urate act to prevent ultraviolet damage in

the integument of the silkworm, *Bombyx mori*. *Archives of insect biochemistry and physiology*, **83**, 41-55.

Klok, C.J. & Harrison, J.F. (2013) The Temperature Size Rule in Arthropods: Independent of Macro-Environmental Variables but Size Dependent. *Integrative and Comparative Biology*, **53**, 557-570.

Kvam, E. & Dahle, J. (2003) Pigmented Melanocytes Are Protected Against Ultraviolet-A-Induced Membrane Damage. *Journal of Investigative Dermatology*, **121**, 564-569.

Laurila, A., Lindgren, B. & Laugen, A.T. (2008) Antipredator defenses along a latitudinal gradient in *Rana temporaria*. *Ecology*, **89**, 1399-1413.

Lindstedt, C., Lindström, L. & Mappes, J. (2009) Thermoregulation constrains effective warning signal expression. *Evolution*, **63**, 469-478.

Macagno, A.L., Pizzo, A., Rolando, A. & Palestrini, C. (2011) Size and shape interspecific divergence patterns partly reflect phylogeny in an *Onthophagus* species-complex (Coleoptera: Scarabaeidae). *Zoological Journal of the Linnean Society*, **162**, 482-498.

Mason, H.S. (1948) The chemistry of melanin III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. *Journal of Biological Chemistry*, **172**, 83-99.

Meinhardt, M., Krebs, R., Anders, A., Heinrich, U. & Tronnier, H. (2008) Wavelength-dependent penetration depths of ultraviolet radiation in human skin. *Journal of Biomedical Optics*, **13**, 044030-044030-5.

Meng, J.-Y., Zhang, C.-Y. & Lei, C.-L. (2010) A proteomic analysis of *Helicoverpa armigera* adults after exposure to UV light irradiation. *Journal of insect physiology*, **56**, 405-411.

Meng, J.-Y., Zhang, C.-Y., Zhu, F., Wang, X.-P. & Lei, C.-L. (2009) Ultraviolet light-induced oxidative stress: Effects on antioxidant response of *Helicoverpa armigera* adults. *Journal of Insect Physiology*, **55**, 588-592.

Minelli, A. (2015) Grand challenges in evolutionary developmental biology. *Frontiers in Ecology and Evolution*, **2**, 85.

Moczek, A.P., Hunt, J., Emlen, D.J. & Simmons, L.W. (2002) Threshold evolution in exotic populations of a polyphenic beetle. *Evolutionary Ecology Research*, **4**, 587.

Mosse, I. & Lyakh, I. (1994) Influence of melanin on mutation load in *Drosophila* populations after long-term irradiation. *Radiation research*, **139**, 357-359.

Murkin, J. & Arango, M. (2009) Near-infrared spectroscopy as an index of brain and tissue oxygenation. *British journal of anaesthesia*, **103**, i3-i13.

Nygren, G.H., Bergström, A. & Nylin, S. (2008) Latitudinal body size clines in the butterfly *Polyommatus icarus* are shaped by gene-environment interactions. *Journal of Insect Science*, **8**, 47.

Ortonne, J.P. (2002) Photoprotective properties of skin melanin. *British Journal of Dermatology*, **146**, 7-10.

Partridge, L. (1987) Life history constraints on sexual selection. *Sexual Selection: Testing the Alternatives* (ed. by J. Bradbury and M. Andersson). John Wiley, London, UK.

Partridge, L. & Coyne, J.A. (1997) Bergmann's rule in ectotherms: is it adaptive? *Evolution*, **51**, 632-635.

Pfeifer, G.P. (1997) Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment. *Photochemistry and photobiology*, **65**, 270-283.

Pont, A.C. & Meier, R. (2002) *The Sepsidae (Diptera) of Europe [electronic resource]*. Brill.

Puniamoorthy, N., Blanckenhorn, W.U. & Schaefer, M.A. (2012a) Differential investment in pre- vs. post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). *Journal of Evolutionary Biology*, **25**, 2253-2263.

Puniamoorthy, N., Schaefer, M.A. & Blanckenhorn, W.U. (2012b) Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). *Evolution*, **66**, 2117-2126.

R Development Core Team (2015) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Reichle, D. (1968) Relation of Body Size to Food Intake, Oxygen Consumption, and Trace Element Metabolism in Forest Floor Arthropods. *Ecology*, **49**, 538-542.

Sang, W., Ma, W.-H., Qiu, L., Zhu, Z.-H. & Lei, C.-L. (2012) The involvement of heat shock protein and cytochrome P450 genes in response to UV-A exposure in the beetle *Tribolium castaneum*. *Journal of Insect Physiology*, **58**, 830-836.

Schiesari, L., Peacor, S.D. & Werner, E.E. (2006) The growth–mortality tradeoff: evidence from anuran larvae and consequences for species distributions. *Oecologia*, **149**, 194-202.

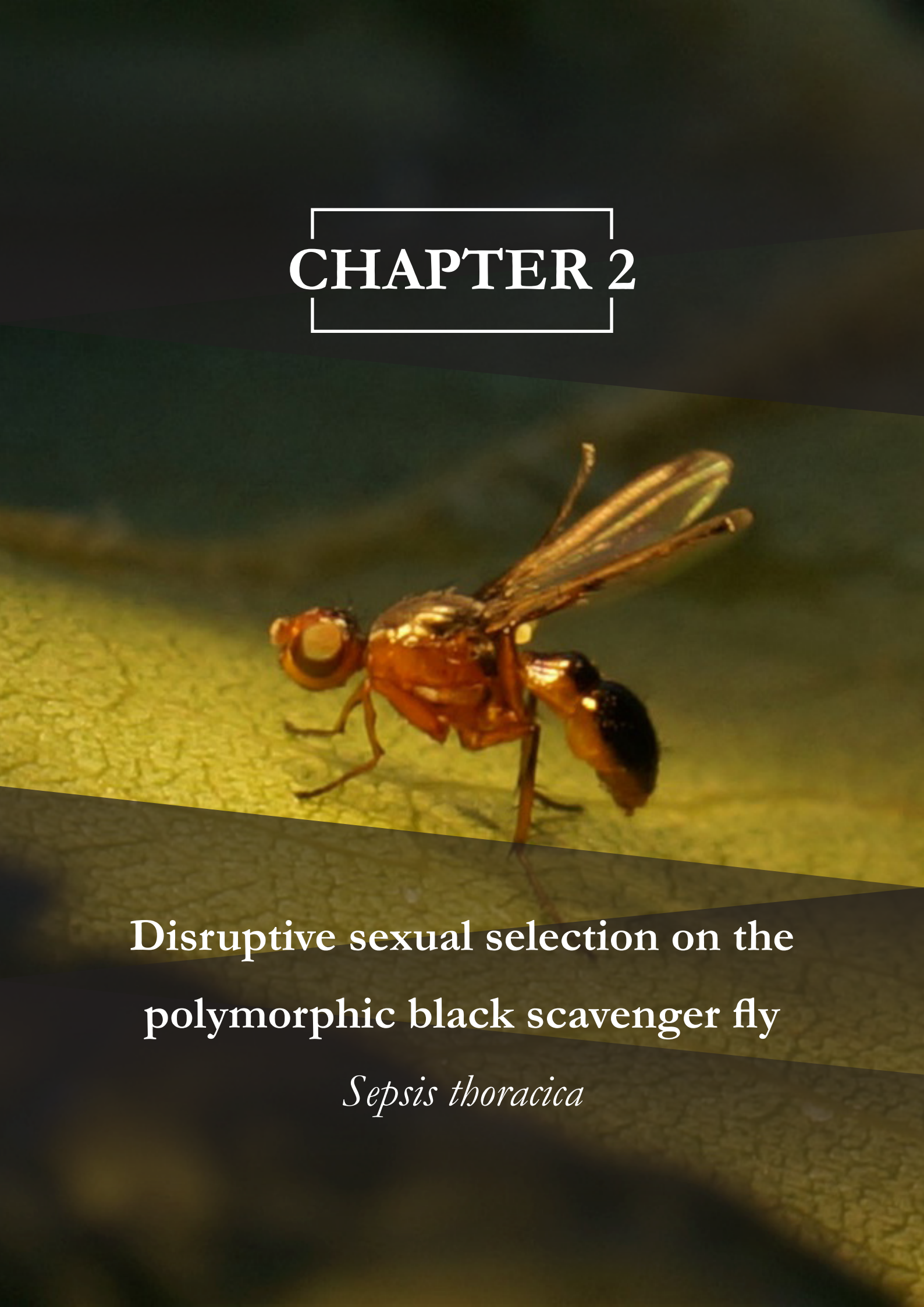
Schmid-Hempel, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, 357-366.

Sgrò, C.M., Terblanche, J.S. & Hoffmann, A.A. (2016) What Can Plasticity Contribute to Insect Responses to Climate Change? *Annual review of entomology*, **61**, 433-451.

Shoval, O., Sheftel, H., Shinar, G., Hart, Y., Ramote, O., Mayo, A., Dekel, E., Kavanagh, K. & Alon, U. (2012) Evolutionary Trade-Offs, Pareto Optimality, and the Geometry of Phenotype Space. *Science*, **336**, 1157-

1160.
Solensky, M.J. & Larkin, E. (2003) Temperature-induced variation in larval coloration in *Danaus plexippus* (Lepidoptera: Nymphalidae). *Annals of the Entomological Society of America*, **96**, 211-216.
Svanbäck, R. & Eklöv, P. (2011) Catch me if you can—predation affects divergence in a polyphenic species. *Evolution*, **65**, 3515-3526.
Taborsky, M. & Brockmann, H.J. (2010) Alternative reproductive tactics and life history phenotypes. *Animal Behaviour: Evolution and Mechanisms* (ed. by P. Kappeler), pp. 537-586. Springer Berlin Heidelberg.
Tada, M., Kohno, M. & Niwano, Y. (2010) Scavenging or quenching effect of melanin on superoxide anion and singlet oxygen. *Journal of clinical biochemistry and nutrition*, **46**, 224-228.
Talloen, W., Dyck, H.V. & Lens, L. (2004) The cost of melanization: Butterfly wing coloration underenvironmental stress. *Evolution*, **58**, 360-366.
Wasserthal, L.T. (1975) The rôle of butterfly wings in regulation of body temperature. *Journal of Insect Physiology*, **21**, 1921-1930.
Watt, W.B. (1969) Adaptive significance of pigment polymorphisms in *Colias* butterflies, II. Thermoregulation and photoperiodically controlled melanin variation in *Colias eurytheme*. *Proceedings of the National Academy of Sciences*, **63**, 767-774.
Whitman, D.W. & Vincent, S. (2008) Large size as an antipredator defense in an insect. *Journal of Orthoptera Research*, **17**, 353-371.
Willmer, P. & Unwin, D. (1981) Field analyses of insect heat budgets: reflectance, size and heating rates. *Oecologia*, **50**, 250-255.
Zuo, W., Moses, M.E., West, G.B., Hou, C. & Brown, J.H. (2012) A general model for effects of temperature on ectotherm ontogenetic growth and development. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 1840-1846.

CHAPTER 2



Disruptive sexual selection on the polymorphic black scavenger fly

Sepsis thoracica

CHAPTER 2

Manuscript Status: To be Submitted
Status in Journal: To be Submitted

Disruptive sexual selection on the polymorphic black scavenger fly *Sepsis thoracica*

Juan Pablo Busso [a], Wolf. U. Blanckenhorn [a]

[a] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zürich

ABSTRACT

Sexual selection has two main components (female preference and male-male competition) that can lead males to adopt alternative reproductive tactics to optimize their reproductive success. Two traits that can significantly influence reproductive success are body size and coloration, since they can facilitate access to females through male contests or as female attractors. We investigated whether, and if so which mechanism of sexual selection contributes to the maintenance and possibly even the establishment of two almost discrete male morphs in the polymorphic black scavenger fly Sepsis thoracica (Diptera: Seaside): small and black (obsidian), or large and yellow (amber). We performed two selection experiments where we registered the mating success of the different male morphs and the behaviors (of both males and females) presumably mediating their mating success. We found evidence for intraspecific disruptive sexual selection on male body size that is mediated by the male-male interactions. This disruptive selection likely contributes to the origin and/or maintenance of the two male morphs in this species. We additionally found significant positive linear selection on body size that interacted with (linear) selection on coloration, probably also contributing to the origin and/or maintenance of the threshold relationship between the two traits. The simultaneous occurrence of disruptive selection and polymorphism in S. thoracica supports the role of sexual selection in the intraspecific diversification of coupled traits (here body size and coloration), which could be a speciation starting point.

KEYWORDS

body size, female preference, male-male competition, melanism, threshold, trade-off

INTRODUCTION

Intraspecific phenotypic diversity can be significantly influenced by sexual selection (Gray and McKinnon, 2007). In some animals, sexual selection not only mediates the evolution of sexual dimorphism (Fairbairn et al., 2007), but also dimorphism within one sex (Hurtado-Gonzales et al., 2010; Rios-Cardenas et al., 2007). In many cases, polymorphisms are maintained by divergent selection between populations, while there is little support for disruptive selection within a population (Gray and McKinnon, 2007). Nevertheless, at least in theory disruptive sexual selection can promote sympatric speciation, a mode of speciation under controversy (Gavrilets and Hayashi, 2005; Gray and McKinnon, 2007; van Doorn et al., 2004).

One of the main components of sexual selection is male-male competition (reviewed by Andersson, 1994; Hunt et al., 2009). Strong competition for mates can lead males to adopt different ways to obtain mates, resulting in alternative reproductive tactics (Oliveira et al., 2008). Gaining privileged access to mates bears costs; as resources are limited, individuals should tailor their investment into different traits so as to optimize their reproductive success through either alternative tactic. When these differential investments are mutually exclusive, they can result in intrasexual polymorphisms (Taborsky and Brockmann, 2010). Such

polymorphisms are then further favored by disruptive selection, reducing the fitness of intermediate phenotypes (Brockmann et al., 2008; Danforth and Desjardins, 1999).

The other main component of sexual selection is mate choice, most commonly exerted by females (Andersson and Simmons, 2006; Hunt et al., 2009). Females are regularly choosier because they face higher reproductive costs than males (Bateman, 1948; Clutton-Brock, 1988). A strong female preference can cause divergence in male phenotypes, which can drive males to adopt alternative reproductive tactics, either to invest in costly structures for mate attraction (bourgeois tactic), or omit these costs and exploit the investment of their bourgeois conspecifics (parasitic tactic) (Oliveira et al., 2008). Females typically prefer bourgeois males, thereby exerting intersexual selection on them, while the parasitic males circumvent such preferences (Gross, 1991; Shuster and Sassaman, 1997; Watson and Simmons, 2010). Consequently, female preference can result in disruptive sexual selection when the males' differential investment partition their morphospace (Taborsky and Brockmann, 2010). Furthermore, female choice can be variable, for instance when females prefer different traits in different males, which can also result in disruptive sexual selection (Busso and Davis Rabosky, 2016; Greene et al., 2000; Sappington and Taylor, 1990; Stelkens et al., 2008).

Nevertheless, male-male competition and female preference are not mutually exclusive such that they can act simultaneously in a species (Hunt et al., 2009). Body size is one of the most important traits that is often favored by classic male–male competition or female choice (reviewed by Andersson, 1994; Blanckenhorn, 2000; Fairbairn et al., 2007; Hunt et al., 2009). Larger body sizes normally facilitate access to females either through victory in aggressive contests between males and/or by forcing copulations (Anderson and Fedak, 1985; Clutton-Brock, 1988; Partridge and Farquhar, 1983; Shine and Mason, 2005; Zucker and Murray, 1996). Larger body sizes are also often favored by female preferences (Brown et al., 1996; MacLaren and Rowland, 2006; Simmons, 1992). These benefits of body size in mate acquisition are particularly evident in species that display alternative reproductive tactics (Dominey, 1980; Emlen, 1997; Taborsky and Brockmann, 2010). In such species, discrimination against intermediate body sizes can result in disruptive selection on male body size (Danforth and Desjardins, 1999).

Another trait that also plays an important role in sexual selection is coloration (Andersson, 1994; Lozano, 1994). Coloration can influence male-male competition when it functions as a signal to other males (Pärt and Qvarnström, 1997; Pérez I de Lanuza et al., 2013). It can also influence female preference when it functions to attract females (Kodric-Brown, 1985; Lozano, 1994), or it can be a simultaneous signal for both sexes (Kodric-Brown, 1996; Pérez I de Lanuza et al., 2013). Through either of these mechanisms, therefore, disruptive selection on coloration can also result in polymorphisms (Greene et al., 2000; Sappington and Taylor, 1990).

Studies focusing on polymorphic species typically address the mechanisms that (stably) maintain the morphs in nature, such as negative frequency dependent selection (Kokko et al., 2007; Maynard Smith, 1982; Zajitschek and Brooks, 2008). We here address the role of disruptive sexual selection as a force widening the intraspecific phenotypic differences between morphs. We focus on the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae), which presents two male morphs: a small, black (obsidian) and a large, yellow (amber) morph (Busso et al, 2017). In other sepsid species, mating success is strongly influenced by the size of the individuals and can involve both female preference and male-male competition (Blanckenhorn et al., 2000; Ward, 1983). By analyzing both male and female behaviors during mating encounters, we here investigated whether, and if so which mechanism of sexual selection contributes to the maintenance and possibly even the establishment of the two male morphs in *S. thoracica*. If male-male competition is more important, we expected significant behavioral differences between the morphs, whereas if female preferences play a greater role, we expected female rejection responses to differ between male morphs. We further assessed if the male polymorphism of *S. thoracica* represents alternative reproductive tactics.

MATERIALS AND METHODS

Fly maintenance and rearing

We sampled 7 European *S. thoracica* populations from Pehka, Estonia (59.48° N, 26.37° W), Ludwigshafen, Germany (49.48° N, 8.42° W), Asturias, Spain (43.3° N, 6.0° E), Petroia, Italy (43.23° N, 12.56° W), Padula, Italy (40.34° N, 15.66° W), Lagonero, Italy (40.14° N, 15.75° W) and Lamezzia, Italy (38.92° N, 16.25° W). Wild-caught females were brought to the laboratory and used to establish multiple (5–25) replicate iso-female line cultures per population that were housed in separate plastic containers at 18±1°C under a 14:10 h light:dark cycle. The cultures were regularly supplied with fresh cow dung, sugar, and water ad libitum.

To generate flies for our sexual selection

experiments, containers with dung were placed for 24 hours inside any of the *S. thoracica* stock cultures for females to deposit eggs. This dung was incubated afterwards in another container until experimental adult flies emerged. To guarantee virginity, females and males were separated upon emergence under a stereo microscope MZ12 (Leica). All flies used in our experiments were 3 to 7 days old.

Estimation of sexual selection in the laboratory

For each experimental trial, we placed 4 amber and 4 obsidian males into a transparent plastic arena (10x10x20 cm) and then introduced 4 females. All flies of one group stemmed from the same isofemale line, but over time a total (N = 90) trials were performed with flies from many different lines, in random order. Based on preliminary trials, all behavioral interactions were observed until all females were paired or otherwise stopped after 20 minutes.

We scored, as proxies for male-male competition, the number of fights and wavings between individual males. We noted the morph of the performer and receiver of these actions, as well as the number of individuals of each morph present at the time of each action. A fight was defined as a male jumping on another male and knocking him over to the ground, while waving consisted of simultaneous wing and abdomen movements directed to another individual. We also measured the mounting latency (time elapsed until the male mounted a female to initiate copulation, without getting off again), the number of mounting attempts (until copulation took place or the trial ended), copulation latency (time elapsed until copulation started), and copulation duration (if it occurred; always measured fully even beyond the 20 min threshold).

As a proxy for female preference we measured female reluctance (time elapsed since the male mounted the female until she bended the abdomen upward allowing him to contact her genitalia). In other sepsids one can measure female shaking as a proxy for female preference (e.g. Blanckenhorn et al., 2000), but we only observed few shaking events in our 90 trials, hence this variable was excluded from further analyses. We tracked which male copulated with which female by isolating the mating pairs from the group.

After the experiment finished, we registered which individuals had copulated and measured the body size of all mated and non-mated individuals. We scored the foreleg femur area, which is an excellent proxy for both body size and coloration, based on previous analyses. All flies were dissected to photograph their foreleg under a stereo microscope MZ12 with a DFC490 camera (Leica) against a neutral white background. The camera was calibrated with a mini IT-8 calibration target to guarantee color consistency between the different pictures. We used a self-written code in ImageJ that measured the amount of pixels of the foreleg femur, and converted them to the real area according to the scale corresponding to each picture. We decided to address coloration variation by measuring melanism, since the morphs vary from amber to black. The code also measured the amount of melanic pixels in the femur, guaranteeing an objective quantification of melanism. The melanic color of the flies was defined as any pixel with a V value over 163 in the YUV colorspace. This threshold was selected based on the valley in the V-value distribution present in the femur coloration (Busso et al. 2017). Melanism represented the proportion of black and brown pixels over all the pixels of the femur.

Alternative reproductive tactics experiment

We performed a second experiment to further quantify behavioral differences between the male morphs (N = 174). Before each trial, both males were marked with a small dot of paint on the back of their thorax to distinguish them when belonging to the same morph. In this experiment, for each trial we placed the males (two ambers, two obsidians or one of each) in a transparent plastic arena (10x10x20 cm) and then we introduced the female. The order of male introduction was chosen at random. During the trials we scored the number of fights and wavings between the males. We also registered the sex and morph of the performer and receiver of these actions. Additionally, we registered mounting attempts, mounting latency, copulation latency, and copulation duration (as above). In this experiment we did not measure the body size of the individuals. Interactions were observed for 20 minutes or until the female was paired.

Statistical Analyses

To assess the intensity of sexual selection on body size and melanism in *S. thoracica,* we used standardized regression methods to generate uni- and bivariate linear and quadratic (non-linear) selection coefficients (Arnold and Wade, 1984; Lande and Arnold, 1983). Univariate coefficients integrate direct and indirect selection on the traits, while bivariate coefficients indicate the selection on one trait controlling for selection on another. Hence, controlling for selection on other traits permits disentangling selection on multiple traits even when they are correlated, and also shows the resulting direction of selection acting on each trait (Arnold and Wade, 1984; Lande and Arnold, 1983). To allow

direct comparison we converted absolute to relative mating success (*z*-scores). We calculated, separately for each trial, standardized Z-scores for body size and melanism of *S. thoracica* by subtracting the trial mean from each value and dividing the difference by the standard deviation: . Relative fitness in each trial was calculated as the dichotomous absolute fitness (i.e., mated or not [1 or 0]) divided by the trial mean fitness (Arnold and Wade, 1984). We used models of relative fitness on *z*-scored body size and melanism, , to estimate univariate linear selection coefficients for each variable, where *w* is the relative fitness, β_1 is the univariate linear selection coefficient, and *z* is the standardized independent variable (body size or melanism). *c* is the intercept in all models. To estimate bivariate linear selection, we used the model , were β_{2bs} and β_{2m} are the bivariate linear selection coefficients of body size and melanism respectively, and z_{bs} and z_m are the standardized body size and melanism. For the univariate quadratic selection we used the model , where γ_1 is half the univariate quadratic selection coefficient; and for the bivariate quadratic selection differentials we used the model , where γ_{2bs} and γ_{2m} are half the bivariate quadratic selection coefficients of body size and melanism respectively, and $\gamma_{2bs \times m}$ is the correlational selection between the two traits. The linear terms of the bivariate equations are not interpreted, as these equations with higher order terms serve to measure only how selection influences the variances and covariances of traits when linear selection effects are controlled (Lande and Arnold, 1983). Significance testing was performed using the corresponding full binomial models described above with binary mating success as the outcome. We also incorporated in the model a random effect of line nested within populations, to control for fly relatedness, and also the trials as random effect, to control for differences between the trail conditions

In the sexual selection experiment, because males of the same morph were hard to differentiate, we calculated fights and wavings per morph. We analyzed the fighting or waving differences between the morphs with a generalized mixed effect model, fitting a negative binomial regression to the data. The negative binomial fitted the data better than the Poisson model and solved the over-dispersion problem. We included in the model as factors the morph of the performer, the morph of the receiver and the interaction between them (N = 90 trials). We also incorporated in the model the random effects of line nested within populations and the trials, as above (Briffa et al., 2013). The model also contained the following offsets: duration of the trial, number of performers (number of individuals of each morph that could perform an action), and number of receivers (number of individuals of each morph that could receive the action). These offsets controlled for the observational time and also for the individuals present when an action was recorded, hence the output is directly interpretable as rates per minute, performer, and receiver (Reitan and Nielsen, 2016). Fights and wavings were analyzed analogously but separately.

For the mounting attempts we fitted a Poisson model, where the variables included were male size, female size, morph and all possible two-way interactions between them. We also incorporated in the model line nested within populations and trials as random effects. The offset in this model was the mounting latency, or alternatively trial duration if there was no copulation. We took mounting latency whenever the male mounted the female and this lead to copulation, in which case the mated pair did not interact anymore with the other males. There were only two cases where another male took over a female, so these trials were excluded from the analyses. To evaluate the rest of the behavioral variables, i.e. mounting latency, copulation latency, copulation duration, and female reluctance (N = 248 mating events), we employed a linear mixed effect model including the same explanatory variables and random effects as in the model for mounting attempts while excluding the offset.

To check for assortative mating, we fitted a linear mixed effect model where the outcome variable was relative male size (standardized per trial) and the predictor variables were relative female size (standardized per trial), male morph, and the interaction between the two. We also incorporated in the model line nested within populations and trial as random effects (N = 248).

For the alternative reproductive tactics experiments, we also used a generalized mixed effect model, fitting a negative binomial regression to the fights and wavings (as above), including the factors morph of the performer, morph of the receiver, and the interaction between them (N = 87 trials). We also included the color marking to detect if it influenced the fly's behavior. The models also contained the random effects of line nested within population and trial. The offset in this case was mounting latency or trial duration, as above. For the mounting attempts, again we fitted a generalized mixed effect model with a Poisson distribution and the same fixed effects, random effects and offsets as for wavings and fights. To evaluate the rest of the behavioral variables, i.e. mounting latency, copulation latency, and copulation duration (N = 24 matings), we employed a linear mixed effect model, including the same explanatory variables and random effects as the model for mounting attempts excluding in these models the offset.

We also analyzed the influence of the fighting and waving behaviors on copulation success with a generalized mixed effect model, fitting a logistic binomial regression to the data. The model included the predictor variables fighting, waving, morph, opponent's morph, marking, and the bivariate interactions fighting:morph, waving:morph, fighting:opponent's morph, and waving:opponent's morph. This model also contained the random effects line nested within population and trial. The offset in this case was again mounting latency or trial duration. All analyses were done using the software R Version 3.2.2 (R Core R Development Core Team, 2015).

RESULTS

Male-male interactions

The morphs have starkly different body sizes (Fig.1, X^2= 1106.386, df = 1, p <0.001). In the sexual selection experiments amber males fought more than obsidians (Fig.2a, X^2= 6.901, df = 1, p =0.009), and obsidians attacked more other obsidians than ambers (*fighter:receiver* X^2=4.213, df = 1, p =0.040), which resulted in both morphs receiving roughly equal numbers of attacks (*receiver* X^2=3.084, df = 1, p =0.080). Ambers also waved more than obsidians (Fig.2c, X^2= 5.581, df = 1, p =0.018), waving equally to both morphs, while obsidians waved mostly to other obsidians (*receiver* X^2= 28.734, df = 1, p<0.001; *signaler:receiver* X^2= 20.188, df = 1, p <0.001).

In the alternative reproductive tactics experiment, we also saw that ambers fight more than obsidians (Fig. 2b, X^2= 6.115, df = 1, p = 0.013), but they were indifferent to the morph of the opponent (*receiver* X^2=0.038, df = 1, p=0.846; *fighter:receiver* X^2= 0.481, df=1, p =0.488). The marking did not significantly affect attacking behavior (X^2= 2.390, df = 1, p =0.122). For the wavings, results differed slightly from the sexual selection experiment. Ambers waved more than obsidians (Fig. 2d, X^2= 11.380, df = 1, p < 0.001), but they waved more to obsidians than to ambers, while the opposite was true for obsidians (*receiver* X^2= 0.828, df = 1, p =0.363; *signaler:receiver* X^2=6.400, df=1, p=0.011). Again, the marking did not significantly affect the waving behavior (X^2= 0.0084, df=1, p=0.923).

We found that, of all variables and bivariate interactions originally included in the model, fighting was the only trait that significantly affected the morphs' copulation success (Fig. 3, X^2= 4.362, df 1, p =0.037); all other variables were not significant (wavings, X^2=1.549, df = 1, p = 0.213; morph, X^2=0.486,

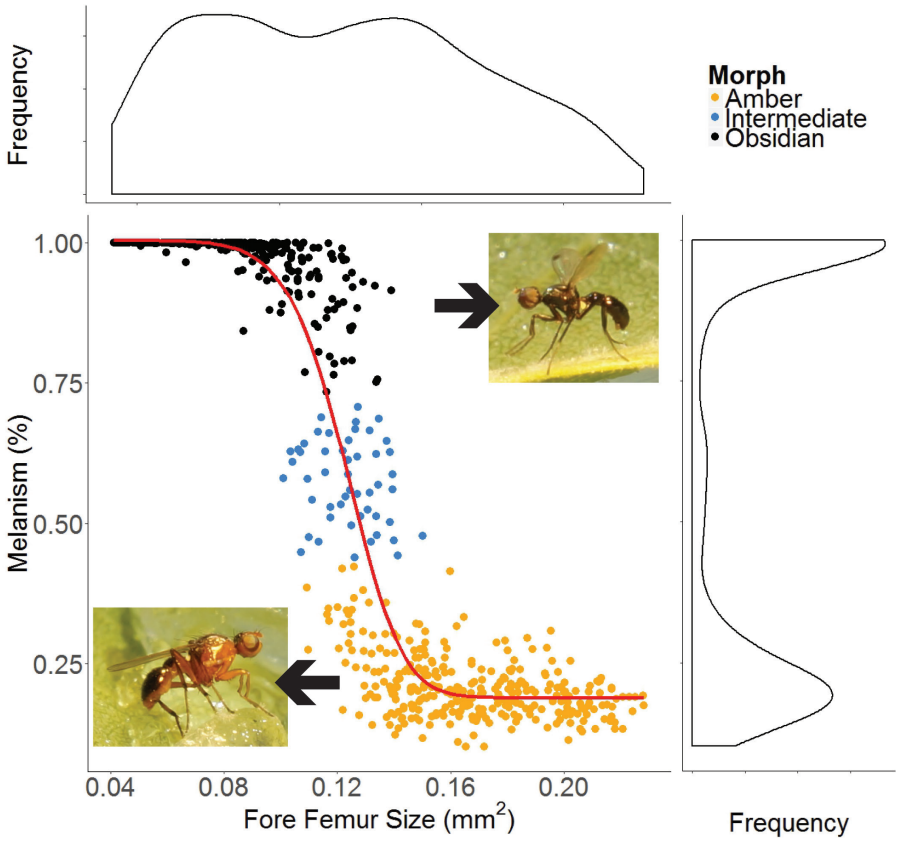


Figure 1. Relationship between body size and melanization of male *Sepsis thoracica*, and their respective frequency distributions. The pictures show the amber and obsidian male morphs.

Table 1. Mean ± 95% CI estimates for all male-female interactions in the sexual selection experiment.

Variables	Morph	Male size	Female Size	Morph:Male size	Morph:Female size	Male size:Female size
Mounting Latency (s)	-866.2 (±1378.8)	-776.1 (± 634.7)	-9560.1 (±6669.1)	792.8 (± 420.6)	10487.5 (± 11745.5)	6169.4 (±5776.4)
Mounting attempts	2.893 (±10.341)	0.909 (±1.635)	0.110 (±1.863) e^{8}	0.327 (±0.363)	0.261 (±7.764) e^{4}	3.006 (±4.607)
Copulation latency (s)	-1048.3 (±1306.4)	-621.8 (±598.9)	-8618.0 (±6280.3)	697.0 (±397.1)	12190.6 (±11119.8)	5148.8 (±5455.5)
Copulation duration (s)	513.21 (±649.22)	173.61 (±285.11)	5231.26 (±3079.71)*	75.37 (±195.76)	-4593.07 (±5462.46)	-1971.29 (±2609.71)
Female reluctance (s)	-168.3 (±326.9)	154.2 (±156.6)	1018.6 (±1592.9)	-67.96 (±99.86)	1550.7 (±2796.2)	-1125.1 (±1386.4)

Table 2. Mean ± 95% CI estimates for all male-female interactions in the alternative reproductive tactics experiment.

Variables	Actor morph	Opponent morph	Marking	Actor:Opponent
Mounting Latency (s)	22.782 (±108.84)	-157.65 (±96.22)	-4.616 (±72.386)	224.81 (±160.06)
Mounting attempts	-0.651 (±0.447)*	0.340 (±0.418)	0.226 (±0.292)	-0.098 (±0.637)
Copulation latency (s)	72.429 (±77.542)	-95.367 (±78.843)	3.853 (±54.461)	-36.533 (±112.65)
Copulation duration (s)	145.17 (±241.13)	-31.19 (±167.89)	45.94 (±145.38)	-331.78 (±282.30)

Table 3. Selection coefficients ± 95% CI for body size and melanism.

Body size				Melanism				Body size x Melanism
$\beta 1_{bs}$	$\beta 2_{bs}$	$\gamma 1_{bs}$	$\gamma 2_{bs}$	$\beta 1_{m}$	$\beta 2_{m}$	$\gamma 1_{m}$	$\gamma 2_{m}$	$\beta 2_{bs} * \beta 2_{m}$
0.537 (±0.144)***	1.019 (±0.442)*	-0.052 (±0.388)	2.068 (±1.900)*	-0.454 (±0.146)**	0.510 (±0.442)	-0.356 (±0.634)	0.764 (±1.790)	-1.791 (±1.750)*

β1: Univariate linear selection coefficient, **β2**: Bivariate linear selection coefficient, **γ1**: Univariate quadratic selection coefficient, and **γ2**: Bivariate quadratic selection coefficient. (n = 710) * P < 0.05; ** P< 0.01; *** P< 0.001.

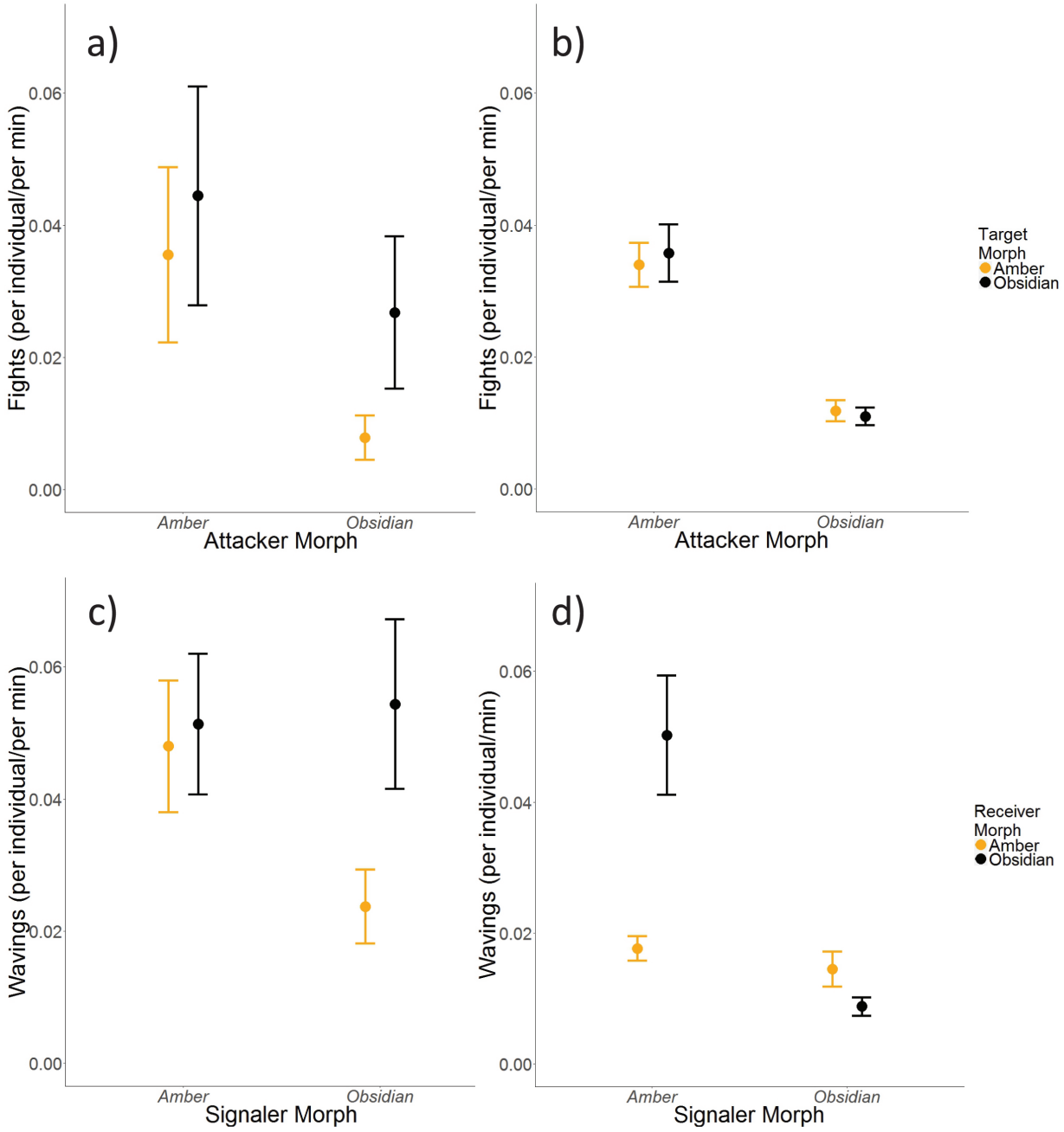


Figure 2. Mean ± 95% CI of fighting and waving frequencies in the sexual selection (a,c) and the alternative reproductive tactics (b,d) experiments.

df = 1, p=0.489; opponent's morph, X^2=2.795, df=1, p = 0.095; marking, X^2= 0.289, df = 1, p = 0.591; morph:opponent's morph, X^2= 0.084, df = 1, p = 0.771; fights:morph, X^2=0.231, df=1, p = .631; wavings:morph, X^2= 1.468, df = 1, p = 0.226; fights:opponent's morph, X^2= 1.384, df = 1, p=0.239; wavings:opponent's morph, X^2= 0.491, df = 1, p=0.484).

Male-female interactions

In the sexual selection experiment we found no assortative mating, since standardized male size was not significantly correlated with standardized female size (X^2= 0.005, df = 1, p =0.942), nor an interaction with morph (X^2= 1.372, df = 1, p =0.504).

The estimates for all variables involved in the male-female interactions are shown in Table 1 for the sexual selection experiment, and in Table 2 for the alternative reproductive tactics experiment. The only significant effect was female body size influencing copulation duration. Nevertheless, this variable depends also on male sperm transfer capabilities. Since the rest of the female behavioral variables (female reluctance and copulation latency) were non-significant, we conclude that the contribution of female choice to sexual selection is minor in this species, at least based on the variables we considered.

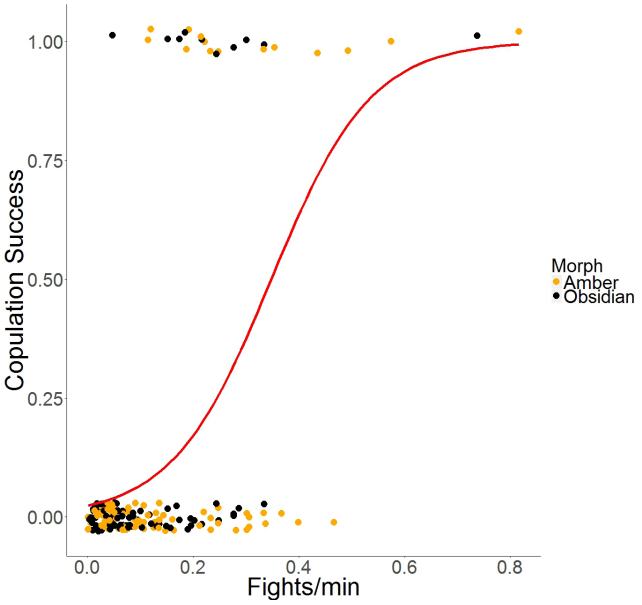


Figure 3. Sigmoid positive influence of fighting on the mating success of the individual.

Selection differentials

Table 3 lists all univariate and bivariate linear and quadratic selection coefficients. To further illustrate the complex numerical interpretation of these coefficients, Fig. 4 depicts the fitness landscape on the males' morphospace resulting from the model including these parameters.

DISCUSSION

In our study we provide the first evidence of ongoing disruptive sexual selection on body size in Diptera. In *S. thoracica*, this disruptive selection was also accompanied by positive linear body size selection, which interacted positively with coloration selection. It is plausible that the combination of disruptive and linear selection forces contributes to the origin and/or maintenance of the two male morphs, although this cannot be proven. The two male morphs, obsidian (smaller black males) and amber (larger orange males), also differ quantitatively (but not qualitatively) in their male-male behaviors (Fig. 2). These behavioral differences, together with the lack of clear female preferences for either morph, suggest that male-male competition is the main underlying mechanism mediating disruptive sexual selection in *S. thoracica*.

We found evidence of disruptive selection only on male body size, selecting against intermediate body sizes. It is therefore likely that the intraspecific male polymorphism in *S. thoracica* either originated and/or is at least maintained by disruptive selection, reducing the fitness of intermediate phenotypes (Brockmann et al., 2008; Danforth and Desjardins, 1999). The mating success of *S. thoracica* males shows a reduction in the fitness of the intermediate phenotypes, such that the bivariate non-linear coefficient $\boldsymbol{\gamma 2}_{bs}$ for body size is negative (Table 3, Fig. 4), which concurs with the phenotypic subdivision of the males' morphospace (Fig. 1). A strong influence of body size on mating success has also been demonstrated in related sepsid flies lacking male polyphenism (Puniamoorthy et al., 2012a; Puniamoorthy et al., 2012b), supporting the general idea that selection on body size has strong effects on the morphospace of the entire clade (Rohner et al., 2016).

Accompanying disruptive sexual selection we find two peaks in the males' fitness landscape (Fig. 4). Strong phenotypic plasticity allows *S. thoracica* males to encompass both peaks in the same genotype. This polyphenism could allow the species to avoid being trapped in either local optimum of the fitness landscape (Woodcock and Higgs, 1996). Ambers occupy a higher fitness peak than obsidians (Fig. 4), which results in overall positive linear sexual selection for larger body sizes, which is often observed in many other animals as well (Blanckenhorn, 2000). It could be that the combination of disruptive and linear selection in *S. thoracica* mediates the escape from the lower (obsidians) to the higher optimum (ambers). Nevertheless, phenotypic plasticity will delay this escape as particular combinations of genetic changes are required (Phillips, 1996; Weissman et al., 2009). This problem can be circumvented by large population sizes, easily achieved by sepsids (Pont and Meier, 2002), that would increase the probability of these genetic combinations to appear. Plasticity thus can facilitate the course of evolution by allowing a species to first explore the entire fitness landscape to then possibly later shift to a higher fitness peak through a combination of disruptive and linear selection.

Strong (directional and disruptive) selection on body size here was however not accompanied by equally strong selection on melanism, which is overall weak. The fact that the significant negative univariate directional melanism selection disappears in the bivariate model documents the indirect influence of body size selection. This is further evinced by their significantly positive correlational selection (Table 3). Correlational selection can explain why the fitness peaks are located at different melanism values (Fig. 4). This interaction of body size and melanism selection, together with disruptive selection on body size, could have contributed to the existing trade-off between body size and melanism observed in *S. thoracica* (Fig. 1; Busso et al., 2017). However, whether it is cause or consequence cannot be determined retrospectively in principle.

The dichotomous male melanism in *S. thoracica* could have also evolved as a badge of status to signal

the body size and fighting ability of an individual (Rohwer, 1982). The honesty of the amber badge could have been maintained by the costs it incurs in terms of energy, physical injury and/or death (Geist, 1974; Georgiev et al., 2013). Moreover, the amber coloration results in a reduced immunocompetence of the individual (Busso et al., 2017). These costs would favor a sigmoid relationship with a trait threshold below which exhibiting the amber color would be highly detrimental in a highly competitive environment (Rohwer, 1982). Consequently, only individuals who are large enough should afford the amber badge, producing linkage between body size and melanism. If a signal is an accurate predictor of the resource holding potential of an individual, threshold traits can be adaptive (Svennungsen et al., 2011). The bimodal fitness landscape in *S. thoracica* encompassing a trade-off between body size and melanism (Busso et al., 2017) supports the idea that disruptive selection favors threshold traits (Svennungsen et al., 2011). The larger body size of ambers results in a mating advantage, while the higher melanism of the obsidians prompts a better immune system (Busso et al., 2017). Since resources are usually limited and these investments are mutually exclusive, this trade-off can contribute to the discontinuity of the fitness optimum resulting in a bimodal fitness landscape (Chevin and Lande, 2013). Hence, it is likely that the interaction between selection forces on body size and melanism contribute to the origin and/or maintenance of the two male morphs in *S. thoracica*.

Accompanying the morphological differences between morphs, we also found divergence in their behavior. In both experiments ambers fought significantly more than obsidians, and the higher fighting frequency increased these males' mating success (Fig. 3). This mating advantage of higher aggressiveness could be either because males fight directly for the females, or because they fight for territories on the dung pats that grant them access to females when the latter come to feed on the dung (personal observation). Through either or both mechanisms our results concur with the higher mating success of aggressive individuals in other species (Brown et al., 2007; Dow and von Schilcher, 1975; Gerlai et al., 2000; Spence et al., 2008).

Nevertheless, aggression also incurs costs in

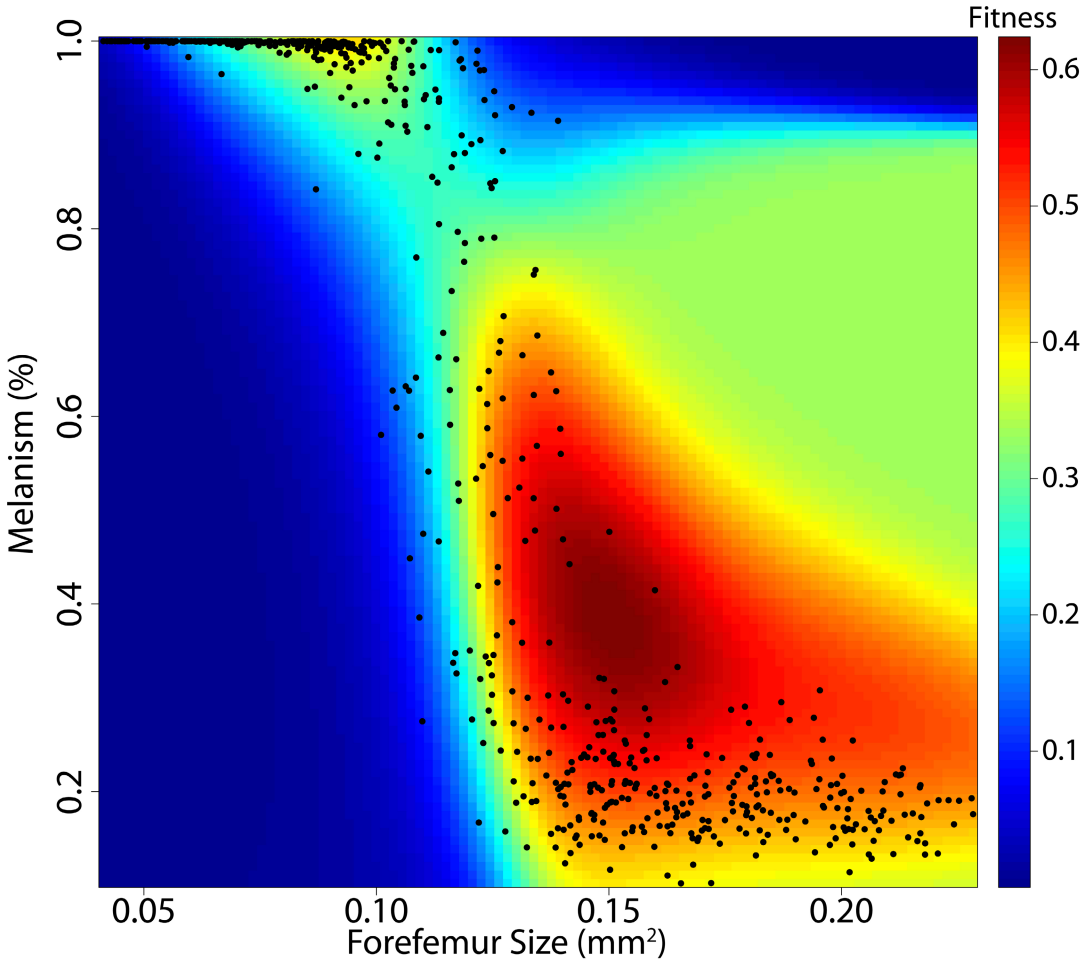


Figure 4. Two-dimensional fitness landscape for the *S. thoracica* males. The black dots represent the actual individual phenotypes.

terms of energy, physical injury or death (Geist, 1974; Georgiev et al., 2013). Hence, individuals might employ signals as an alternative way to limit these fighting costs (Parker, 1974; Rohwer, 1982; Tinbergen, 1951). We found that ambers waved more than obsidians in both experiments, which seem to result in less fighting and waving from obsidians to ambers at least in the sexual selection experiment (Fig. 2). However, waving did not significantly influence the mating success of the males. This lack of effect could be either because waving is not an honest signal of the individual's resource holding potential, or because it has another meaning. Alternatively, the presence of females could have influenced the waving behavior in form of an audience effect (Zuberbühler, 2008), which can alter the original message of the signal. Furthermore, since we observed some variation of the waving behavior between the two experiments, it could be that the signals are dependent on the environmental setting (Endler, 1992; Schluter and Price, 1993). Further experiments are needed to understand the signaling behavior of sepsid flies.

Regardless, there needs to be a mechanism regulating the frequency of the two morphs so that both can coexist in the long term. A threshold trait employing body size seems to be an adequate solution to obtain frequency dependent selection of evolutionary stable strategies, since juvenile population density is typically negatively correlated with body size (Busso et al., 2017). At low densities the species can afford to produce only aggressive amber males ("hawks"; cf. Parker, 1974) because the risks of fighting will be lower. On the other hand, as density increases the fighting risks also increase and only the most competitive individuals will be able to bear the amber badge, which deters fights with obsidians. At very high densities the majority, if not all, individuals will be less aggressive obsidians ("doves"). Hence a threshold could provide a mechanism to balance the ratio between the two morphs and render optimal combinations of ambers (hawks) and obsidians (doves) according to the population density to minimize their costs.

In contrast to male competitive behavior, female preferences did not play a role in this species, according to the behaviors we registered. We found no specific female preferences for any morph, coloration or body size in the sexual selection experiment. This lack of preference was observed for all female mating behaviors studied (copulation latency and duration, female reluctance to mate). Hence, our combined evidence suggests that male-male competition is the primary diversification agent in this species (Seehausen and Schluter, 2004), also contributing to the male-biased sexual size dimorphism exhibited by the amber morphs (Rohner et al., 2016).

In our study we estimated fitness benefits based on individual mating success, but as mentioned the amber morph may incur other costs outside of mating such as energy loss, physical injury or death (Geist, 1974; Georgiev et al., 2013), which should influence survival. Additionally, body size affects fitness by influencing a large set of interrelated life-history traits (Blanckenhorn, 2000). Further studies encompassing other fitness components are the logical next step towards understanding the possible role of nonlinear selection on maintaining the male polymorphism in *S. thoracica*. Predation pressure certainly additionally affects the costs and benefits of the morphs (Busso & Blanckenhorn, 2017 – Chapter 5).

To conclude, we found evidence for intraspecific disruptive sexual selection on male body size in the dimorphic fly *S. thoracica* that is mediated by the male-male interactions, as also seen in dung beetles (Moczek and Emlen, 1999; Moczek and Emlen, 2000). This disruptive selection likely contributes to the origin and/or maintenance of the male morphs in this species, together with size-selective predation selection (Busso & Blanckenhorn 2017) and possibly other processes. We additionally found significant positive linear selection on body size that interacted with coloration, probably also contributing to the origin and/or maintenance of the threshold relationship between the two traits. The morphological and behavioral differences between the morphs indicate at least the beginning of alternative reproductive strategies in *S. thoracica*. To our knowledge this is the first study showing intraspecific disruptive sexual selection in Diptera, adding an important contribution to an otherwise short list of examples (Busso and Davis Rabosky, 2016; Greene et al., 2000; Sappington and Taylor, 1990; Stelkens et al., 2008). The simultaneous existence of disruptive selection and polymorphism in *S. thoracica* supports the role of sexual selection in the intraspecific diversification of traits (Andersson, 1994; Eberhard, 2010), which could be a speciation starting point (Corl et al., 2010; Wittkopp et al., 2009). The fact that disruptive selection acts entirely within a species is of particular interest because it could strengthen reproductive isolation and sympatric speciation by sexual selection, a controversial phenomenon in evolutionary biology (Gavrilets and Hayashi, 2005; van Doorn et al., 2004).

REFERENCES

Anderson SS, Fedak MA, 1985. Grey seal males: energetic and behavioural links between size and sexual success. Animal Behaviour 33:829-838.

Andersson B, 1994. Sexual Selection: Princeton University Press.

Andersson M, Simmons LW, 2006. Sexual selection and mate choice. Trends in Ecology & Evolution 21:296-302.

Arnold SJ, Wade MJ, 1984. On the measurement of natural and sexual selection: applications. Evolution:720-734.

Bateman AJ, 1948. Intra-sexual selection in *Drosophila*. Heredity 2:349-368.

Blanckenhorn WU, 2000. The evolution of body size: What keeps organisms small? Q Rev Biol 75:385-407.

Blanckenhorn WU, Mühlhäuser C, Morf C, Reusch T, Reuter M, 2000. Female Choice, Female Reluctance to Mate and Sexual Selection on Body Size in the Dung Fly *Sepsis cynipsea*. Ethology 106:577-593.

Briffa M, Hardy IC, Gammell MP, Jennings D, Clarke DD, Goubault M, 2013. Analysis of animal contest data. Animal contests:47e85.

Brockmann HJ, Oliveira R, Taborsky M, 2008. 20· Integrating mechanisms and function: prospects for future research. Alternative Reproductive Tactics An Integrative Approach:471.

Brown WD, Chimenti AJ, Siebert JR, 2007. The payoff of fighting in house crickets: motivational asymmetry increases male aggression and mating success. Ethology 113:457-465.

Brown WD, Wideman J, Maydianne CBA, Mason AC, Gwynne DT, 1996. Female Choice for an Indicator of Male Size in the Song of the Black-Horned Tree Cricket, *Oecanthus nigricornis* (Orthoptera: Gryllidae: Oecanthinae). Evolution 50:2400-2411.

Busso JP, Davis Rabosky AR, 2016. Disruptive selection on male reproductive polymorphism in a jumping spider, *Maevia inclemens*. Animal Behaviour 120:1-10.

Chevin L-M, Lande R, 2013. Evolution of Discrete Phenotypes from Continuous Norms of Reaction. The American Naturalist 182:13-27.

Clutton-Brock TH, 1988. Reproductive success: studies of individual variation in contrasting breeding systems: University of Chicago Press.

Corl A, Davis AR, Kuchta SR, Sinervo B, 2010. Selective loss of polymorphic mating types is associated with rapid phenotypic evolution during morphic speciation. Proceedings of the National Academy of Sciences of the United States of America 107:4254-4259.

Danforth B, Desjardins C, 1999. Male dimorphism in *Perdita portalis* (Hymenoptera, Andrenidae) has arisen from preexisting allometric patterns. Insectes sociaux 46:18-28.

Dominey WJ, 1980. Female mimicry in male bluegill sunfish - a genetic polymorphism. Nature 284:546-548.

Dow MA, von Schilcher F, 1975. Aggression and mating success in *Drosophila melanogaster*. Nature.

Eberhard WG, 2010. Evolution of genitalia: theories, evidence, and new directions. Genetica 138:5-18.

Emlen DJ, 1997. Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). Behavioral Ecology and Sociobiology 41:335-341.

Endler JA, 1992. Signals, signal conditions, and the direction of evolution. American Naturalist 139:S125-S153.

Fairbairn DJ, Blanckenhorn WU, Székely T, 2007. Sex, size and gender roles: evolutionary studies of sexual size dimorphism: Oxford University Press.

Gavrilets S, Hayashi TI, 2005. Speciation and Sexual Conflict. Evol Ecol 19:167-198.

Geist V, 1974. On fighting strategies in animal combat. Nature 250:354-354.

Georgiev AV, Klimczuk AC, Traficonte DM, Maestripieri D, 2013. When violence pays: a cost-benefit analysis of aggressive behavior in animals and humans. Evolutionary psychology 11:147470491301100313.

Gerlai R, Lahav M, Guo S, Rosenthal A, 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacology Biochemistry and Behavior 67:773-782.

Gray SM, McKinnon JS, 2007. Linking color polymorphism maintenance and speciation. Trends in Ecology & Evolution 22:71-79.

Greene E, Lyon BE, Muehter VR, Ratcliffe L, Oliver SJ, Boag PT, 2000. Disruptive sexual selection for plumage coloration in a passerine bird. Nature 407:1000-1003.

Gross MR, 1991. Evolution of Alternative Reproductive Strategies: Frequency-Dependent Sexual Selection in Male Bluegill Sunfish. Philosophical Transactions: Biological Sciences 332:59-66.

Hunt J, Breuker CJ, Sadowski JA, Moore AJ, 2009. Male–male competition, female mate choice and their interaction: determining total sexual selection. Journal of evolutionary biology 22:13-26.

Hurtado-Gonzales JL, Baldassarre DT, Uy JAC, 2010.

Interaction between female mating preferences and predation may explain the maintenance of rare males in the pentamorphic fish *Poecilia parae*. Journal of Evolutionary Biology 23:1293-1301.

Kodric-Brown A, 1985. Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology 17:199-205.

Kodric-Brown A, 1996. Role of male-male competition and female choice in the development of breeding coloration in pupfish (*Cyprinodon pecosensis*). Behavioral Ecology 7:431-437.

Kokko H, Jennions MD, Houde A, 2007. Evolution of frequency-dependent mate choice: keeping up with fashion trends. Proceedings of the Royal Society B-Biological Sciences 274:1317-1324.

Lande R, Arnold SJ, 1983. The Measurement of Selection on Correlated Characters. Evolution 37:1210-1226.

Lozano GA, 1994. Carotenoids, Parasites, and Sexual Selection. Oikos 70:309-311.

MacLaren RD, Rowland WJ, 2006. Differences in female preference for male body size in using simultaneous versus sequential stimulus presentation designs. Behaviour 143:273-292.

Maynard Smith J, 1982. Evolution and the theory of games. Cambridge ; New York :: Cambridge University Press.

Moczek A, Emlen D, 1999. Proximate determination of male horn dimorphism in the beetle *Ontophagus taurus* (Coleoptera: Scarabaeidae). Journal of Evolutionary Biology 12:27-37.

Moczek AP, Emlen DJ, 2000. Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? Animal behaviour 59:459-466.

Oliveira RF, Taborsky M, Brockmann HJ, 2008. The evolution of alternative reproductive tactics: concepts and questions. Alternative Reproductive Tactics: Cambridge University Press.

Parker G, 1974. Assessment strategy and the evolution of animal conflicts. Journal of theoretical biology 47:223-243.

Pärt T, Qvarnström A, 1997. Badge size in collared flycatchers predicts outcome of male competition over territories. Animal Behaviour 54:893-899.

Partridge L, Farquhar M, 1983. Lifetime mating success of male fruitflies (*Drosophila melanogaster*) is related to their size. Animal Behaviour 31:871-877.

Pérez I de Lanuza G, Font E, Monterde JL, 2013. Using visual modelling to study the evolution of lizard coloration: sexual selection drives the evolution of sexual dichromatism in lacertids. Journal of Evolutionary Biology 26:1826-1835.

Phillips PC, 1996. Waiting for a compensatory mutation: phase zero of the shifting-balance process. Genetical Research 67:271-283.

Pont AC, Meier R, 2002. The Sepsidae (Diptera) of Europe [electronic resource]: Brill.

Puniamoorthy N, Blanckenhorn WU, Schaefer MA, 2012a. Differential investment in pre- vs. post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). Journal of Evolutionary Biology 25:2253-2263.

Puniamoorthy N, Schaefer MA, Blanckenhorn WU, 2012b. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). Evolution 66:2117-2126.

Author. 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Reitan T, Nielsen A, 2016. Do Not Divide Count Data with Count Data; A Story from Pollination Ecology with Implications Beyond. PLOS ONE 11:e0149129.

Rios-Cardenas O, Tudor MS, Morris MR, 2007. Female preference variation has implications for the maintenance of an alternative mating strategy in a swordtail fish. Animal Behaviour 74:633-640.

Rohner PT, Blanckenhorn WU, Puniamoorthy N, 2016. Sexual selection on male size drives the evolution of male-biased sexual size dimorphism via the prolongation of male development. Evolution:n/a-n/a.

Rohwer S, 1982. The Evolution of Reliable and Unreliable Badges of Fighting Ability. American Zoologist 22:531-546.

Sappington TW, Taylor OR, 1990. Disruptive sexual selection in *Colias eurytheme* butterflies. Proceedings of the National Academy of Sciences 87:6132-6135.

Schluter D, Price T, 1993. Honesty, perception and population divergence in sexually selected traits. Proceedings of the Royal Society of London Series B-Biological Sciences 253:117-122.

Seehausen O, Schluter D, 2004. Male–male competition and nuptial–colour displacement as a diversifying force in Lake Victoria cichlid fishes. Proceedings of the Royal Society of London B: Biological Sciences 271:1345-1353.

Shine R, Mason RT, 2005. Does large body size in males evolve to facilitate forcible insemination? A study on garter snakes. Evolution 59:2426-2432.

Shuster SM, Sassaman C, 1997. Genetic interaction between male mating strategy and sex ratio in a marine isopod. Nature 388:373-377.

Simmons LW, 1992. Sexual Selection and Body Size in a Natural Population of the Field Cricket, *Gryllus campestris* (L.). Journal of Orthoptera Research:12-13.

Spence R, Gerlach G, Lawrence C, Smith C, 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. Biological Reviews 83:13-34.

Stelkens RB, Pierotti MER, Joyce DA, Smith AM, van der Sluijs I, Seehausen O, 2008. Disruptive sexual selection on male nuptial coloration in an experimental hybrid population of cichlid fish. Philosophical Transactions of the Royal Society of London B: Biological Sciences 363:2861-2870.

Svennungsen TO, Holen ØH, Leimar O, 2011. Inducible Defenses: Continuous Reaction Norms or Threshold Traits? The American Naturalist 178:397-410.

Taborsky M, Brockmann HJ, 2010. Alternative reproductive tactics and life history phenotypes. In: Kappeler P, editor. Animal Behaviour: Evolution and Mechanisms: Springer Berlin Heidelberg. p. 537-586.

Tinbergen N, 1951. The study of instinct.

van Doorn GS, Dieckmann U, Weissing FJ, 2004. Sympatric speciation by sexual selection: a critical reevaluation. The American Naturalist 163:709-725.

Ward PI, 1983. The effects of size on the mating behaviour of the dung fly *Sepsis cynipsea*. Behavioral Ecology and Sociobiology 13:75-80.

Watson NL, Simmons LW, 2010. Mate choice in the dung beetle *Onthophagus sagittarius*: are female horns ornaments? Behavioral Ecology 21:424-430.

Weissman DB, Desai MM, Fisher DS, Feldman MW, 2009. The rate at which asexual populations cross fitness valleys. Theoretical Population Biology 75:286-300.

Wittkopp PJ, Stewart EE, Arnold LL, Neidert AH, Haerum BK, Thompson EM, Akhras S, Smith-Winberry G, Shefner L, 2009. Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila*. Science 326:540-544.

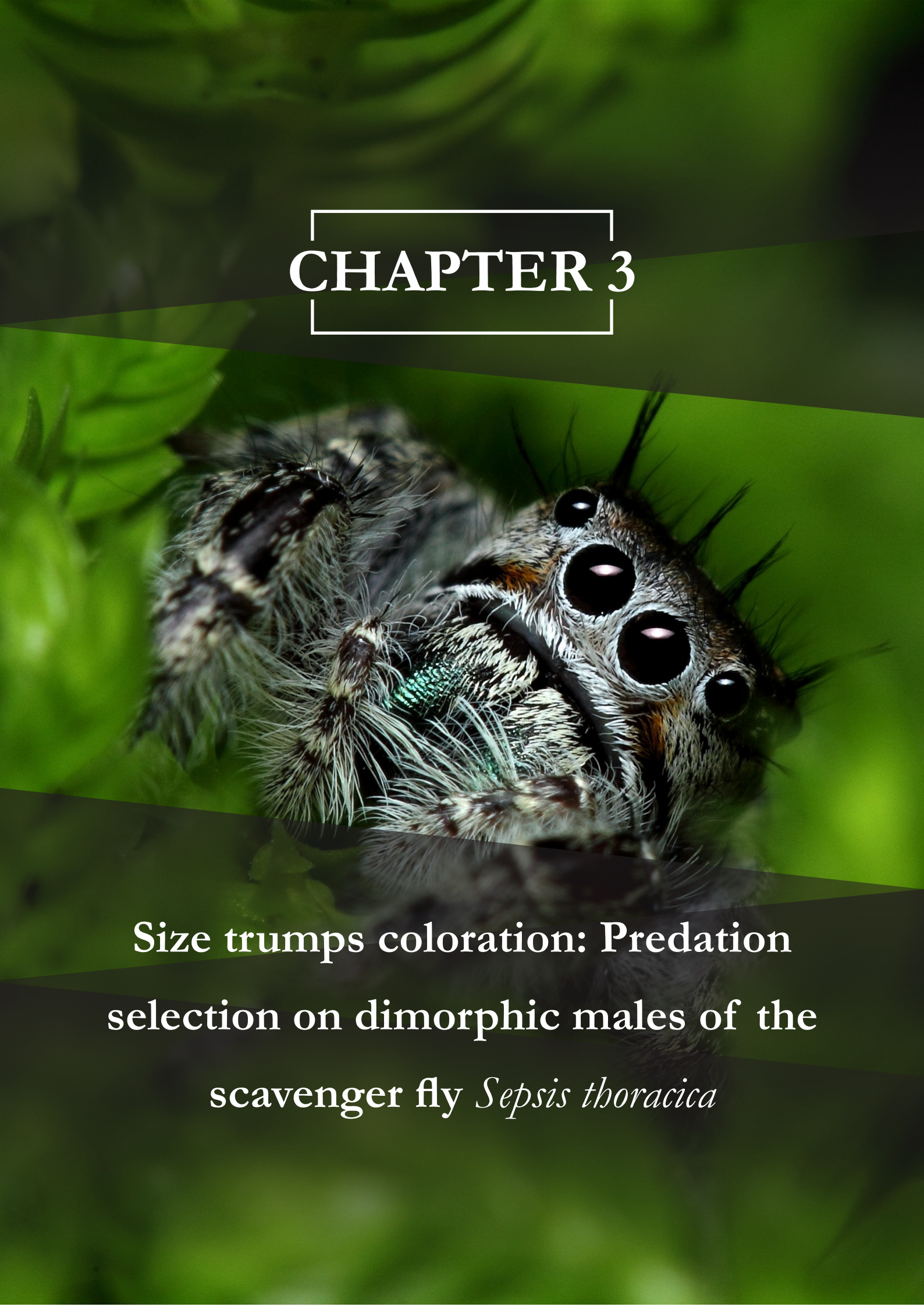
Woodcock G, Higgs PG, 1996. Population Evolution on a Multiplicative Single-Peak Fitness Landscape. Journal of Theoretical Biology 179:61-73.

Zajitschek SRK, Brooks RC, 2008. Distinguishing the Effects of Familiarity, Relatedness, and Color Pattern Rarity on Attractiveness and Measuring Their Effects on Sexual Selection in Guppies (*Poecilia reticulata*). The American Naturalist 172:843-854.

Zuberbühler K, 2008. Audience effects. Current Biology 18:R189-R190.

Zucker N, Murray L, 1996. Determinants of Dominance in the Tree Lizard *Urosaurus ornatus*: the Relative Importance of Mass, Previous Experience and Coloration. Ethology 102:812-825.

CHAPTER 3



Size trumps coloration: Predation selection on dimorphic males of the scavenger fly *Sepsis thoracica*

CHAPTER 3

Manuscript Status: Submitted to Behavioral Ecology
Status in Journal: Major Revisions

Size trumps coloration: Predation selection on dimorphic males of the scavenger fly *Sepsis thoracica*

Juan Pablo Busso [a], Wolf. U. Blanckenhorn [a]

[a] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zürich

ABSTRACT

Predation is a major factor affecting trade-offs and shaping a species' morphospace. Two of the main traits influencing prey survival are body size and coloration. *Sepsis thoracica* males display a sigmoid trade-off relationship between these two traits, defining a size threshold above which investment in melanin drastically drops, resulting in two morphs: a small black (Obsidian) and a large orange morph (Amber). In this study, we used laboratory predation experiments to estimate the intensity of viability selection exerted by different arthropod predators on *S. thoracica* body size and coloration. Selection was performed against two different backgrounds mimicking the natural habitat (dung and grass), for which the camouflage and aposematism of the morphs would vary. Our experiments showed that body size is generally under positive selection and overpowers selection on coloration, which varied significantly among predator species and backgrounds. However, predation does little to modify the relationship between these traits, rather it seems to move the phenotypes right along the sigmoid curve. We conclude that for the black scavenger fly *S. thoracica* predator evasion and escaping skills determined by body size are more effective against invertebrate predators than its conspicuousness determined by coloration, contrasting what has been found for vertebrate predators (mostly birds), where prey coloration is important and there is negative selection on size. Because arthropod predators generally have strong effects on insect populations, the positive directional selection imposed by invertebrate predators is likely an important force driving the evolution of body size in *S. thoracica* and insects in general.

KEYWORDS

Predation, Natural selection, trade-off, polymorphism, Sepsidae, body size, coloration, spectral sensitivity, Diptera, sigmoid.

INTRODUCTION

Predation is a major factor affecting trade-offs and shaping a species' morphospace (reviewed by Ruxton et al., 2004). Avoiding predation involves numerous traits, but since resources are usually limiting, a trade-off between defense traits is likely to evolve to optimize the prey's survival (Mikolajewski et al., 2006; Vencl and Srygley, 2013). Survival probability varies with each particular combination of defense traits (Domenici et al., 2008; Vamosi and Schluter, 2004) and also with the predator (Abrams, 2000). Two of the main traits drastically influencing the survival probability of prey individuals are body size (Berger et al., 2006; Whitman and Vincent, 2008) and coloration (Svanbäck and Eklöv, 2011). For example, an increase in body size can increase the success of escaping an attack (Dayton et al., 2005; Langerhans, 2009; Lundvall et al., 1999; Van Buskirk and Schmidt, 2000; Whitman and Vincent, 2008). This advantage mainly derives from larger sized prey having greater strength and better defenses against the particular handling skills of a given predator (Brönmark and Miner, 1992; Schmitt and Holbrook, 1984; Shine, 1991; Whitman and Vincent, 2008). On the other hand, larger body size augments the energetic value of the prey for the predator (Norberg, 1978), thus directly affecting the predators' feeding preference (Dixon and Hemptinne, 2001; Gaston et al., 1997).

Another factor influencing the prey's survival probability is its coloration (Svanbäck and Eklöv, 2011). Among insects, a widespread strategy to intraspecifically alter coloration is through changes in the melanin production (Cook and Saccheri, 2013; True, 2003), often resulting in camouflage or aposematism (Guilford, 1990). The level of camouflage determines the success of avoiding detection and being attacked (Feltmate and Williams, 1989; Hargeby et al., 2004; Johannesson and Ekendahl, 2002). Changes in coloration can thus affect survival probability by decreasing the detectability of the prey (Fabricant and Herberstein, 2015). In contrast, aposematism is a warning signal displayed by prey to signal its unpalatability to potential predators, making individuals more conspicuous but ultimately reducing their probability of being attacked (Poulton, 1890; see Ruxton et al., 2004 for further references and review). This warning effect can be enhanced by larger body size (Nilsson and Forsman, 2003), hence coloration and body size can have combined effects on the survival probability of the prey. This will likely involve a trade-off, because body size itself is resource-demanding (Reichle, 1968), and melanin is also costly to synthesize (Freitak et al., 2005; Hooper et al., 1999; Talloen et al., 2004). Consequently, individuals need to balance their investment into each trait to optimize their survival probability against various predators.

An excellent system to address how predation influences the putative trade-off between body size and coloration is *Sepsis thoracica* (Diptera: Sepsidae). Males of this species exhibit extreme variability in size and color, with smaller males being mostly black and the largest males bright orange, while females are completely black (Pont and Meier, 2002). This male coloration could play a role in sexual selection for attracting mates, the attraction of predators being a concomitant negative consequence (Andersson, 1994; Houde, 1994; Partridge, 1987). Alternatively, this coloration could be aimed at predators, since males feed and wait for the females on and around dung pats (Pont and Meier, 2002). Black color will camouflage them against the background, while orange color will make them conspicuous on dung. Therefore, the orange coloration could either be a very risky reduction in camouflage (on the dung), or a very clear aposematic warning signal. In a meadow full of flowers, in contrast, orange may blend in better than black coloration. Hence, *S. thoracica* presents a unique opportunity to analyze the influence of predation on the phenotypic trade-off between coloration and body size and distinguish the role of coloration for aposematism and camouflage.

However, sepsid flies face not only one, but a wide range of predators (Parker, 1972; Tuomikoski, 1952). We thus performed laboratory predation selection experiments in two backgrounds using three predators of different arthropod guilds to investigate how prey body size and coloration affect *S. thoracica* survival. We focus on invertebrate predators because they can have stronger effects than vertebrates especially on small insects (Fagan and Hurd, 1994; Kristensen, 1994; Lang et al., 1999; Wooster, 1994). The size gap between *S. thoracica* and invertebrate predators is much reduced relative to that with vertebrate predators; hence we hypothesized that body size would influence the survival probability against invertebrate predators mostly by affecting predator evasion or escaping skills. We thus expected that survival probability would generally increase with prey size, but that there would be differences in this relationship among the various arthropod predators due to their different hunting strategies and handling skills. Alternatively, the detectability and energetic value of body size could play a greater role in predation, in which case survival probability would decrease with body size, varying among predators according to their differing energetic requirements and handling capabilities. We also investigate the response to selection by asking whether viability selection by arthropod predators in any way alters the relationship between body size and coloration in this species.

We were also interested on the influence of aposematism and camouflage on invertebrate predators, because most of the aposematism research has used vertebrates (mainly birds) as predators (reviewed by Ruxton et al., 2004). In arthropods, there is evidence of unlearned avoidance towards red, yellow and green items, suggesting an innate component towards avoiding colors typically involved in aposematism (Taylor et al., 2014). Therefore, we hypothesized that the black coloration would be important for camouflage on the dung, in which case we expected that survival probability would decrease with the orangeness of the prey. Alternatively, the orange color could have an aposematic role and it would be a very clear warning signal against predators, predicting that survival probability would increase with the orangeness. The hypotheses regarding body size and coloration are not mutually exclusive, as two or more effects can act simultaneously on the system.

We used standardized univariate and bivariate selection methodology to measure the intensity and variation of viability selection on body size and coloration between predators (Arnold and Wade, 1984; Lande and Arnold, 1983). To distinguish between camouflage and aposematism we performed the selection experiments against two different backgrounds (dung and grass), in which the camouflage and aposematic significance of the fly coloration

would vary. We also analyzed how the predators' selection pressure affected the body size-coloration trade-off by comparing the trait relation before and after predation. This design allowed us to disentangle the effects of prey body size and coloration on survival for the different predators and backgrounds.

METHODOLOGY

Sepsis thoracica *maintenance and rearing*

We sampled eight European *S. thoracica* populations along a latitudinal gradient from Ludwigshafen, Germany (49.48º N, 8.42º W), Nordrach, Germany (49.4º N, 8.08º W), Zürich, Switzerland (47.34º N, 8.54º W), Asturias, Spain (43.3º N, 6.0º E), Petroia, Italy (43.23º N, 12.56º W), Padula, Italy (40.34º N, 15.66º W), Lamezzia, Italy (38.92º N, 16.25º W), Adrano, Italy (37.67º N, 14.83º W). Wild-caught females were brought to the laboratory and used to establish cultures of multiple (10–20) replicate iso-female lines per population that were housed in separate plastic containers and regularly supplied with fresh cow dung, sugar, and water ad libitum. The cultures were maintained at 18 ± 1 °C, under a 14:10 h light:dark cycle.

Predator collection and maintenance

We sampled the arthropod community in grassland and forest edges around the University of Zurich's Irchel campus using sweep netting to collect predators that co-occur with the sepsids. Each predator individual was placed alone in a container with several *S. thoracica* individuals in order to identify the species that feed on *S. thoracica*. From those species feeding on the *S. thoracica*, we selected the ones with the highest abundances in the sampled environments to guarantee a strong effect of their predation pressure on the sepsid flies. We ultimately selected three visually oriented predators (Harland and Jackson, 2000; Land, 1969a, b; Parker, 1965, 1969; Teuschl et al., 2010; Williams and McIntyre, 1980) from three different guilds and of different body sizes so that the prey's coloration and background likely play a role for prey survival. We chose the jumping spider *Heliophanus flavipes* (Areanea: Salticidae), an active hunter between 6-9 mm long (Nentwig et al., 2016), the damsel bug *Himacerus mirmicoides* (Heteroptera: Nabidae), a sit-and-wait predator between 11-14 mm long with polyphagous feeding habits (Lattin, 1989), and the predatory dung fly *Scatophaga stercoraria* (Diptera: Scathophagidae), an active hunter between 6-12 mm long (Teuschl et al., 2010). All these predators suck out the content of their prey, leaving the exoskeleton behind, which allowed us to measure the morphological traits of the captured prey after predation. To eliminate potential sex biases, we used only females as predators, which as a rule should be more voracious.

The spider and bug predators collected in the field were maintained individually in plastic containers and provided with water in cotton-stoppered vials and fed regularly with a mix of *Drosophila* species (*D. melanogaster, D. virilis, D. americana* and *D. novamexicana*). Individuals were used in the predation experiments only after they had reached their adult stage. All *S. stercoraria* individuals were raised in the laboratory.

Predation experiment

We used as prey a mix of *S. thoracica* flies from random lines and random populations to reduce any possible effect of local adaption to predators. To raise the prey flies, a container with more or less dung, to generate individuals of varying sizes, was placed for 24 hours inside any of the *S. thoracica* stock cultures for females to deposit eggs. This dung was incubated afterwards in a novel container to obtain adult prey males to be used in our predation trials.

Prior to the trials each predator was kept in abstinence for 5 days, and water was provided regularly. Right before starting the trials, the jumping spiders and the bugs were photographed dorsally under a stereo microscope MZ12 with a DFC490 camera (Leica). We measured the body size of the predator from digital photographs using ImageJ. For the salticids we used the area of the cephalothorax as a proxy of body size, for the bugs the area of their pronotum, and for the *S. stercoraria* flies hind tibia length. For the *S. thoracica* prey individuals we measured the area of the foreleg femur and its coloration, using a self-written code that measured the amounts of orange and black pixels in the femur.

The predation trials were carried out in a rectangular acrylic arena (10 (W) x 20 (L) x 10 cm (H)). We placed into the arena a randomly selected predator, plus six *S. thoracica* males (three orange and three black) from a random line and population. Each predation container was provided with sugar and water ad libitum. Each trial was also randomly assigned to one of two backgrounds: dung or grass. The backgrounds consisted of a printed photograph of the natural habitat of the flies that covered the four sides of the arena and the bottom, leaving the top clear. The arenas were placed under 58W lamps emitting light in the visual and UV spectrum (FB58, Arcadia) to simulate the natural lighting conditions. The experiments were carried out at 24 ± 1 °C, under

a 16:8 h light:dark cycle. A trial concluded when at least 3 flies had been eaten by the predators (but not all), and we scored survival as a dichotomous variable (alive/dead). After the experiment, we measured coloration and body size of both preyed and surviving flies. There were 21 to 23 replicates per predator-background treatment combination.

Statistical analyses

To assess the intensity of viability selection by predation on body size and coloration of *S. thoracica,* we used standardized regression methods to generate uni- and bivariate linear and quadratic (non-linear) selection coefficients (Arnold and Wade, 1984; Lande and Arnold, 1983). We thus calculated, separately for each trial, standardized Z-scores for body size and coloration of *S. thoracica* by subtracting the trial mean from each value and dividing the difference by the standard deviation: . Relative fitness in each trial was calculated as the dichotomous absolute fitness (i.e., survival [1 or 0]) divided by the trial mean fitness (Arnold and Wade, 1984). We used models of relative fitness on Z-scored body size and coloration, , to estimate univariate linear selection differentials for each variable, where *w* is the relative fitness, β_1 is the univariate linear selection coefficient, and *z* is the standardized independent variable (body size or coloration). The letter *c* is the intercept in all models. To estimate bivariate linear selection, we used the model , were β_{2bs} and β_{2c} are the bivariate linear selection coefficients of body size and coloration respectively, and z_{bs} and z_c are the standardized body size and coloration. For the univariate quadratic selection we used the model , where γ_1 is half the univariate quadratic selection coefficient; and for the bivariate quadratic selection differentials we used the model , were γ_{2bs} and γ_{2c} are half the bivariate quadratic selection coefficients of body size and coloration respectively, and $\gamma_{2bs \times c}$ is the correlational selection between the two traits. Significance testing was performed using the corresponding full binomial model with binary survival as the dependent, predator species and background as fixed factors, prey body size and coloration as well as predator body size as continuous covariates with all relevant interactions, and trial as random effect. Non-significant higher order interactions were removed from the final model. We additionally partitioned the dataset by predator and background to calculate for each treatment combination all the selection coefficients mentioned above.

For the relation between body size and coloration in *S. thoracica* we fitted a 5-parameter logistic regression model: orangeness = (*d*-*c*)/[1+(BS/*e*)^*b*]^*f* , where BS is body size, *d* is the estimated coloration at body size zero, *c* is the estimated orangeness at infinite body size, *e* is the mid-range body size, *b* is the slope at the inflection point, and *f* is the asymmetry factor (Gottschalk and Dunn, 2005).

To measure the effects of selection on the sigmoid relationship between body size and coloration, we compared the general 5-parameter logistic regression fitted to all *S. thoracica* individuals (n = 757; before selection) to another regression fitted just to the surviving individuals (n = 360; after selection). We examined the differences between each of the 5 parameters of both regressions using t-tests. We also did the same for the subsample of flies in each background treatment. All analyses were done using the software R Version 3.2.2 (R Core R Development Core Team, 2015).

RESULTS

The body size of the predators never had any significant effect on survival, either singly or in interactions with prey body size or coloration. We found that there is overall positive directional selection for larger body size by predation in *S. thoracica* in the overall univariate model, i.e. when body size was considered alone (β_{1bs} in Table 1). In this model we also found a significant three-way interaction between prey body size, predator species and background (Fig. 1a and b, likelihood ratio X^2= 7.510, df = 2, p=0.023). However, this is only due to the difference in the slope for the damsel bug *H. mirmicoides* on a grass background (Fig. 1b), since removing this treatment from the dataset rendered the three-way interaction insignificant (likelihood ratio X^2= 3.118, df = 1, p =0.108), while positive directional selection remained significant and unchanged (β_{1bs} = 0.417 ± 0.106, p < 0.001; cf. Table 1). The two-way interaction between body size and background was not significant (likelihood ratio X^2= 0.538, df = 1, p =0.463), nor was the interaction between body size and predator species (likelihood ratio X^2= 2.627, df = 2, p =0.269).

Coloration also showed positive directional selection in the univariate model (β_{1c} in Table 1) as well as a significant interaction with predator species and background (Fig. 1c and d, likelihood ratio X^2= 8.245, df = 2, p =0.016). However, the two-way interactions between coloration and background (likelihood ratio X^2= 1.362, df = 1, p =0.243) and between coloration and predator species (likelihood ratio X^2= 1.219, df=2, p =0.544) were not significant.

In the bivariate model, both body size and coloration were significant (Table 1). The three-way interaction between body size, predator species and background was also significant (likelihood ratio X^2= 7.481, df = 2,

Table 1. Selection coefficients ± Standard Error for body size and coloration.

		Body size				Coloration				Body size x Coloration
Predator	**Background**	$\beta 1_{bs}$	$\beta 2_{bs}$	$\gamma 1_{bs}$	$\gamma 2_{bs}$	$\beta 1_{c}$	$\beta 2_{c}$	$\gamma 1_{c}$	$\gamma 2_{c}$	$\gamma 1_{bs} \times \gamma 1_{c}$
All Predators	All Backgrounds	0.417±0.108***	0.816±0.244***	0.056±0.316	-0.15±0.75	0.354±0.108***	-0.422±0.232*	0.228±0.636	0.236±0.756	0.134±0.081
H. flavipes	Dung	0.417±0.116***	0.733±0.341*	0.056±0.34	0.736±1.282	0.354±0.117***	-0.336±0.341*	0.228±0.692	0.966±1.488	-0.712±1.224
	Grass	0.312±0.091***	0.719±0.215***	0.056±0.264	0.678±0.998	0.206±0.093*	-0.448±0.215*	0.014±0.52	0.686±0.962	-0.642±0.838
H. mirmicoides	Dung	0.296±0.1***	0.245±0.238	0.128±0.27	-0.228±0.694	0.279±0.1**	0.056±0.238	0.746±0.49	0.688±0.856	0.203±0.663
	Grass	-0.052±0.111	-0.045±0.231	0.396±0.296	0.362±0.576	-0.047±0.111	-0.008±0.231	0.69±0.39	0.76±0.726	-0.174±0.487
S. stercoraria	Dung	0.219±0.109*	0.133±0.232	0.28±0.264	0.368±0.66	0.215±0.11*	0.098±0.232	0.376±0.414	0.408±0.66	-0.176±0.492
	Grass	0.529±0.112***	0.28±0.255	0.156±0.318	0.018±0.84	0.528±0.112***	0.277±0.255	-0.698±0.616	-1.128±1.03	0.252±0.736

β1: Univariate linear selection coefficient, **β2**: Bivariate linear selection coefficient, **γ1**: Univariate quadratic selection coefficient, and **γ2**: Bivariate quadratic selection coefficient. (n = 757).

Table 2. Review of the absorbance peaks in spectral sensitivity of Diptera

Family	Species	Common name	UV (nm)	Violet (nm)	Blue (nm)	Green (nm)	References
Syrphidae	*Eristalis tenax*	Honeybee mimic	350		450	520	Horridge et al. 1975
Tabanidae	*Tabanus nigrovittatus*	Horse fly		440	480	520	Allan et al. 1991
Muscidae	*Musca autumnalis*	Face fly	360		490		Agee and Patterson 1983
	Haematobia irritans	Horn fly	360		490		Agee and Patterson 1983
Keroplatidae	*Arachnocampa luminosa*	Glowworm	382		460	540	Meyer-Rochow and Eguchi 1984
Tephritidae	*Ceratitis capitata*	Fruit fly	365		485-500		Agee et al. 1982
	Dacus oleae	Olive fly	365		485-500		Agee et al. 1982
	Rhagoletis cerasi	Cherry fruit fly	365		485-500		Agee et al. 1982
Anthomyiidae	*Delia radicum*	Cabbage root fly	340-350		490	546	Brown and Anderson 1996
Calliphoridae	*Phaenicia sericata*	Common green bottle fly	350		480		McCann and Arnett 1972
	Calliphora erythrocephala	Blow fly	350	440		520	Smola and Meffert 1979
Culicidae	*Aedes aegypti*	Yellow fever mosquito	323-345			523	Muir et al. 1992
Drosophilidae	*Drosophila melanogaster*	Common fruit fly	350		470		Stark et al. 1976

p =0.024), as was the interaction between coloration and predator species (likelihood ratio X^2= 7.064, df=2, p =0.029). In this model, the three-way interaction between coloration, predator species and background was not significant (likelihood ratio X^2= 0.190, df = 2, p =0.910), as was the interaction between coloration and background was non-significant (likelihood ratio X^2= 0.007, df = 1, p =0.934). Furthermore, quadratic and correlational selection coefficients were non-significant in all models, including all their interactions (Table 1).

We found a sigmoid relation between body size and coloration in *S. thoracica* across all the eight sampled populations, featuring a unimodal body size and a bimodal coloration distribution (Fig. 2). This sigmoid relation remained unaffected by predation selection in the full dataset or any of the treatment combinations of predator species and background (Supplementary Table 1).

DISCUSSION

We found a positive relationship between the body size of *S. thoracica* male prey and their survival in predation experiments, implying positive directional selection for larger body size by all the three arthropod predators used. Our bivariate model indicates that selection on body size likely is more important than selection on coloration, although this cannot be determined with certainty, as these two traits are highly correlated in this species. *Sepsis thoracica* males display a sigmoid trade-off relationship between body size and coloration, resulting in a body size threshold that divides males into two color morphs: the small and black morph, which we called Obsidian, and the large and orange morph, which we called Amber (Fig. 2). Nevertheless, this sigmoid relationship between the two traits was not significantly affected by the predation selection documented here.

We found that survival of *S. thoracica* was positively correlated with body size in both the univariate (body size only) and the bivariate selection models (body size and coloration). Both uni- and

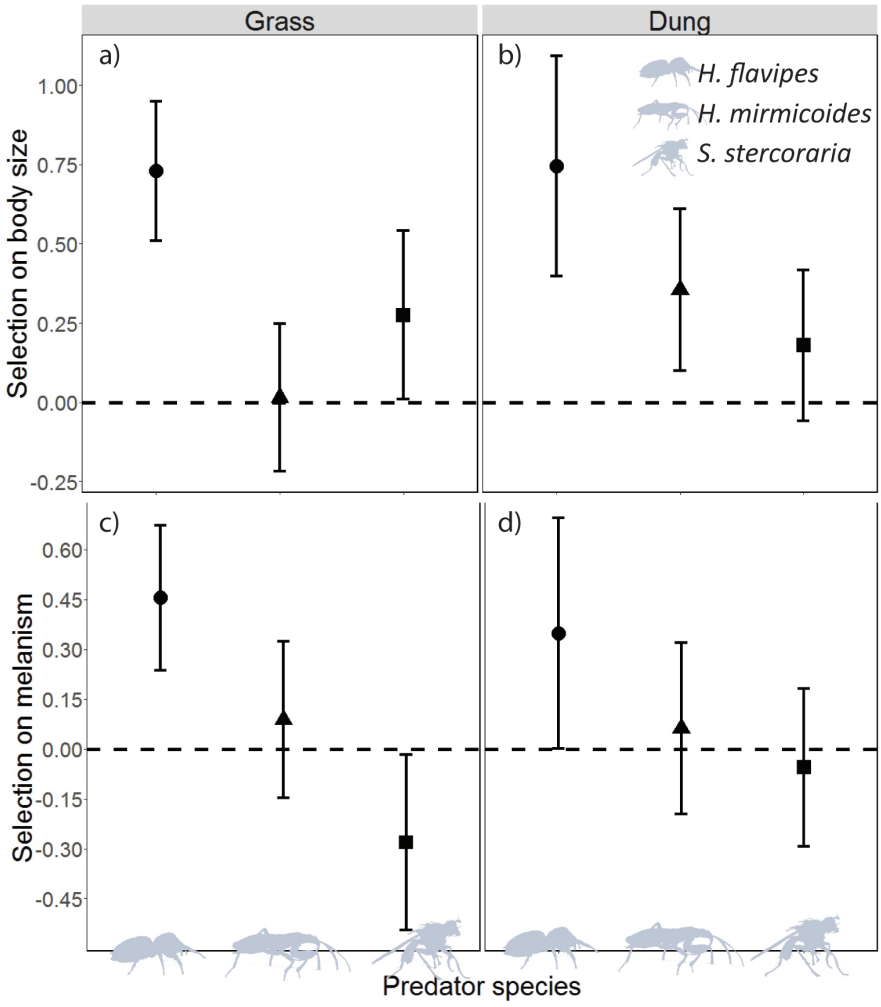


Figure 1. Mean (± SE) bivariate selection differentials for body size (a,b) and coloration (c,d) for each predator in the dung and grass backgrounds.

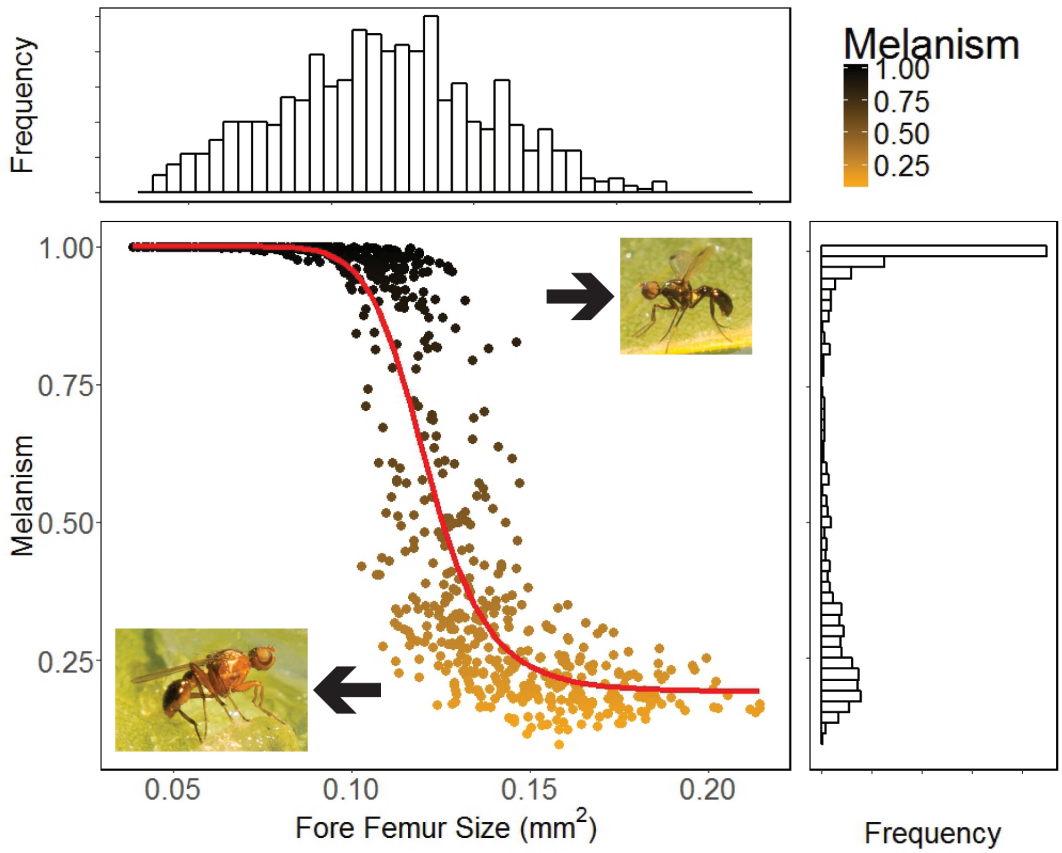


Figure 2. Relationship between body size and coloration in *Sepsis thoracica* males, and their respective frequency distributions.

bivariate selection coefficients are positive (Table 1), the bivariate coefficient being larger. This difference is likely due to counteracting selection on coloration and/or the high correlation between the two traits. Although of different guilds, all three predators exerted positive selection on body size of the prey in both backgrounds. This increase in survival with body size is likely mediated primarily by better prey evasion skills, as strength and size typically increase in parallel, and not so much by the energetic value of the prey for the predator (Bailey, 1986; Cogni et al., 2002; Dixon, 1959; Dixon and Russel, 1972; Griffiths, 1980; Iwasaki, 1991; Mänd et al., 2007; Morris, 1963; New, 1992; Okuyama, 2007; Stortenbeker, 1967; Vermeij, 1982; Wilson, 1975). These results concur with the positive selection on body size by *S. stercoraria* on the closely related *Sepsis cynipsea* (Teuschl et al., 2010). Such positive selection on body size has also been observed in the few studies addressing this issue with invertebrate predators (Ovadia and Schmitz, 2002; Whitman and Vincent, 2008), suggesting that positive selection on prey body size may be a general phenomenon among invertebrate predators whose prey is not much smaller than themselves.

In our study positive selection on *S. thoracica* body size was observed for all predators against all backgrounds, except for one scenario, the damsel bug *H. mirmicoides* in a grass background, where there was no selection on body size. In this treatment, the obsidian morph experienced an equal survival rate than the amber morph. This puzzling result could be due to several different reasons. Firstly, it could be simply that the predator does not have any preference for any body size, since it has polyphagous feeding habits (Lattin, 1989). Alternatively, it could be that the predator evasion strategies used by the larger, amber *S. thoracica* males in the grass background are not efficient against *H. mirmicoides*, while those of the obsidians are. Lastly, it could be that the damsel bug adjusts its feeding habits (Lattin, 1989) to the environmental background. It is thus possible that both prey and predator are responsible for the lack of selection on body size observed in this particular treatment. Nevertheless, this outcome is an exception among our results since selection on body size was overall positive in all other treatments, highlighting the importance of prey body size for various invertebrate predators.

The positive selection on body size found here contrasts with the often negative selection on this trait exerted by vertebrate predators (Churchfield et al., 1991; Curio, 2012; Mattson et al., 1968; Prop, 1960; Schülert and Dicke, 2002; Tinbergen, 1960). This contrast could be due to several reasons. In general the conspicuousness of invertebrate prey to vertebrate predators increases with prey body size (Curio, 2012;

Schülert and Dicke, 2002; Shine and Thomas, 2005; Winfield and Townsend, 1983), primarily due to the concomitant increase in the detection/encounter rate (Curio, 2012; Goerlitz and Siemers, 2007; Maiorana, 1981; Maly, 1970; Troost et al., 2008). Moreover, larger insects have fewer possibilities of finding hiding places (Edmunds, 1974), leaving them generally more exposed. Importantly here, optimal foraging theory (Stephens and Krebs, 1986) and previous empirical studies (Churchfield et al., 1991; Curio, 2012; Mattson et al., 1968; Prop, 1960; Schülert and Dicke, 2002; Tinbergen, 1960) suggest that vertebrate predators such as birds, lizards or amphibians tend to prefer larger prey items because of their higher energetic value (Chen et al., 2004; McCracken et al., 2004; Morin, 1984; O'Brien et al., 1976). This greater relative importance of the energetic value for vertebrate compared to invertebrate predators probably relates to the generally greater body size differences between prey and predator. That is, when facing vertebrates, an increase in *S. thoracica* body size would not much affect their escaping prospect but would considerably increase the energy intake of the predator, whereas when facing invertebrate predators, escaping chances would be significantly augmented by even small body size increases. As a result, body size necessarily experiences conflicting selection pressures in insect prey in nature, at least as regards predation.

In contrast to body size, coloration was under negative selection in the bivariate model, whereas the coefficient resulted positive in the univariate model. This drastic change in sign in the bivariate model indicates that there is some counter-selection against the amber color, but that this selection is overpowered by selection on body size such that the effect of coloration in the univariate model is largely due to its correlation with body size. Nevertheless, there is no strong and consistent correlational selection component evident in the model (Table 1). This greater importance of body size than coloration concurs with what has been observed for folivorous insect larvae vis-à-vis several different invertebrate predators (Dyer, 1997; Dyer and Floyd, 1993; Remmel et al., 2011; Remmel and Tammaru, 2009).

Nevertheless, the two-way interaction between coloration and predator species was significant in the overall bivariate model, as was the three-way interaction between coloration, predator species and background in the overall univariate model for coloration, suggesting that selection on coloration depended on the predator and the background in which this encounter took place (Figures 1c,d). We observe the same trend in both backgrounds, but the differences between predators are greater in the grass background. Selection on coloration was always close to zero in the dung background, meaning that black flies were not better camouflaged nor were amber males more conspicuous. It could be that motion is more important than coloration for prey detection by the predators used, as found for other invertebrate predators (Land, 1971, 1985; Parker, 1965, 1969; Tarsitano and Jackson, 1994; Wheater, 1989). Sepsids are well known for their conspicuous, almost permanent wing-waving behavior (Pont and Meier, 2002), the function of which is unclear but does facilitate their detection.

We did observe some interesting disparities between the predators, particularly in the grass but also the dung background (Fig. 1c,d). Selection against orange color was strong (i.e. negative) for the jumping spider but positive for the yellow dung fly, suggesting that the jumping spider was able to well differentiate the amber morph from the background while the fly likely could not (the bug was indifferent). The compound eyes of insect predators limit their visual resolution and viewing distance (Land, 1997a, b), while salticids have high resolution, single-chambered corneal eyes (Blest et al., 1990; Land, 1969a, b; Land and Nilsson, 2012) with a spatial acuity approaching that of primates and surpassing that of many birds (Harland and Jackson, 2000; Land, 1972, 1974; Williams and McIntyre, 1980). Although normally most groups of insects and spiders lack a receptor in the red part of the spectrum (Briscoe and Chittka, 2001; Théry and Gomez, 2010), which would make amber individuals cryptic against a green grass background (Chittka and Waser, 1997; Fabricant and Herberstein, 2015), there are heteropterans and salticids that can perceive red wavelengths (Schwind et al., 1984; Zurek et al., 2015). These spectral sensitivity differences could explain the different results observed for the three predators against the grass background.

In jumping spiders, a long-pass filter in their retina can shift the sensitivity of a subset of green photoreceptors to red (Zurek et al., 2015). Hence, we can assume that the jumping spider was indeed able to differentiate the amber morph from the green grass background. For the damsel bug, the specific bivariate analyses revealed that there was no selection on color. It has been observed that vision plays an important role in the predation behavior of damsel bugs, and both prey movement and contrast with the background influence predation (Parker, 1965, 1969). It could be that *H. mirmicoides* can perceive the red color as *Notonecta glauca*, another heteropteran, can (Schwind et al., 1984). This would allow *H. mirmicoides* to distinguish the amber morph from the grass background, and not perceive it as monochromatically green, like other invertebrate predators (Briscoe and Chittka, 2001; Chittka and Waser, 1997). Nevertheless,

non-significant selection on coloration for the damsel bug (Table 1) in both backgrounds, would actually suggest that the bug predator has no color preference.

The predatory yellow dung fly females (*S. stercoraria*) showed strongest positive selection in the grass background favoring large amber and disfavoring small black prey individuals (Fig. 1d), while in the dung background they were indifferent as regards color (Fig. 1c). This is difficult to explain. As the males of this species are yellowish-orange themselves (while the females are greyish-green), and females tend to avoid harassment by the larger and very aggressive males, the orange color could indeed be acting as an aposematic signal reducing predation on the amber *S. thoracica* morph. Alternatively, the differences could be caused by the background affecting the detection probability of the prey. As mentioned before, insects normally do not possess a photoreceptor to detect red color (Briscoe and Chittka, 2001), and a brief review of the spectral sensitivity of flies showed that they are no exception (Table 2). If this is also true for *S. stercoraria*, the fly should consequently not be able to distinguish the amber flies from the grass background, augmenting the amber morph's survival probability there. We are not implying that orange cannot be perceived, but rather it would have appeared monochromatically green and would potentially have been difficult to distinguish from a grass background (Chittka and Waser, 1997; Pickens, 1990). These results, in combination with lack of advantage of amber morphs against the dung background, suggest that either the amber coloration is an aposematic signal limited to very particular backgrounds (here grass), or that the amber males were simply not detected by *S. stercoraria*.

Although we found differences between the predators, overall selection on coloration was negative in the bivariate model. This negative value likely results from the strong influence of the negative coloration selection by the jumping spider, as shown by the similarity between the general and species-specific selection coefficients. Nevertheless, coloration selection was positive in the univariate model, which indicates that overall selection on body size overpowers selection on coloration. Hence, it is likely that for *S. thoracica* predator evasion and escaping skills determined by body size are more effective against invertebrate predators than its conspicuousness determined by coloration.

The secondary role of coloration selection by invertebrate predators contrasts with the results for vertebrate (mostly avian) predators, where prey coloration is under strong selection (Marples et al., 2005; reviewed by Ruxton et al., 2004). This contrast can be due to the differences in the visual system in combination with the prey detection mechanism of the different taxa. Most birds have sharp visual acuity and color vision encompassing a wide range of the color spectrum plus ultraviolet frequencies (Jones et al., 2007; Osorio and Vorobyev, 2008), and they often select their prey based on its coloration (reviewed by Stevens, 2007). On the other hand, the visual system of most insects and spiders is more constrained since they are monochromats, dichromats, or UV/blue/green trichromats (Briscoe and Chittka, 2001), and detect their prey mainly through its movement (Land, 1971, 1985; Parker, 1965, 1969; Tarsitano and Jackson, 1994; Wheater, 1989). These discrepancies in the effects on prey body size and coloration by vertebrate and invertebrate predators highlight that the selection pressures exerted by avian predators should not be extrapolated to other taxa.

In *S. thoracica* males, body size and coloration present a sigmoid relationship (Fig. 2), which evinces a complex trade-off involving a body size threshold above which the individuals dramatically decrease their investment in melanin, becoming almost completely orange. Such threshold traits are commonly related to polymorphisms (Taborsky and Brockmann, 2010), as observed e.g. in the horned beetle *Ontophagus acuminatus* (Emlen, 1997) and other dung beetles (Moczek et al., 2002). In *S. thoracica* this threshold divides the males into two morphs: the small and black morph, which we called Obsidian, and the large and amber morph, which we called Amber. Since we found both male morphs in all isofemale lines of all populations, the morphs are not genetically determined but they result from a plastic, condition-dependent polyphenism, which still shows a heritable component (unpublished data, and the subject of another study). Here, we focused on the influence of viability selection by invertebrate predators of different guilds shaping this relationship by analyzing both body size and coloration simultaneously. Although body size was under strong positive and coloration under negative selection, we believe that these selection forces alone cannot explain the size/coloration polymorphism of *S. thoracica* males. In *O. acuminatus* it has been observed that the sigmoid curve can be modified under different selection pressures (Emlen, 1996). We had suspected that the polymorphism of *S. thoracica* males could result from disruptive selection on coloration (Bond and Kamil, 2002; Jackson and Li, 2004; Masta and Maddison, 2002), but in all our models the quadratic components of coloration were never significant. Alternatively, the polymorphism could have resulted from the evolution of the threshold steepness through disruptive selection of the underlying factor, body size (Chevin and Lande, 2013), but in all our selection

models the quadratic components of body size were also non-significant. Furthermore, viability selection by invertebrate predators did also not have any effect on the sigmoid relationship between body size and coloration because the sigmoid curves before and after selection were identical (Supplementary table 1). It could be that the detected selection on body size was not strong enough to reshape the curve, and probably moves the phenotypes right along the sigmoid curve.

In conclusion, we showed that body size is generally under positive selection by various invertebrate predators in this species, but selection on male coloration varied significantly among predator species and with background color. The positive selection on body size and the secondary role of selection on coloration by invertebrate predators contrasts with what has been observed for vertebrate predators, where negative selection on body size predominates together with strong selection on coloration. Nevertheless, the predation pressure by arthropod predators can be substantially greater than that exerted by vertebrates (Loiselle and Farji-Brener, 2002), and several studies have shown that arthropod predators can have strong effects on insect populations (Fagan and Hurd, 1994; Kristensen, 1994; Lang et al., 1999; Wooster, 1994). Consequently, the positive directional selection imposed by arthropods is likely an important force driving the evolution of body size in invertebrates. However, given the multitude of predators on any given prey species, vertebrate or invertebrate, the corresponding net viability selection resulting in the wild can only be assessed comprehensively in a phenomenological way (when taking the prey perspective), but not in a mechanistic or functional manner as done here. We also documented in *S. thoracica* a sigmoid relationship between body size and coloration, defining a threshold above which investment in melanin drastically drops. Although we found positive selection on body size and negative selection on coloration, these forces did not modify the sigmoid curve between body size and coloration. Future studies addressing the influence of other selection forces on this trade-off, such as sexual selection, should shed light onto the evolution and maintenance of this trade-off and resulting male morphs in *S. thoracica*.

REFERENCES

Abrams PA, 2000. Character shifts of prey species that share predators. The American Naturalist 156:S45-S61.

Agee HR, Boller E, Remund U, Davis JC, Chambers DL, 1982. Spectral sensitivities and visual attractant studies on the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), olive fly, *Dacus oleae* (Gmelin), and the European cherry fruit fly, *Rhagoletis cerasi* (L.) (Diptera, Tephritidae). Zeitschrift für Angewandte Entomologie 93:403-412.

Agee HR, Patterson RS, 1983. Spectral Sensitivity of Stable, Face, and Horn Flies and Behavioral Responses of Stable Flies to Visual Traps (Diptera: Muscidae). Environmental Entomology 12:1823-1828.

Allan SA, Stoffolano Jr JG, Bennett RR, 1991. Spectral sensitivity of the horse fly *Tabanus nigrovittatus* (Diptera: Tabanidae). Canadian Journal of Zoology 69:369-374.

Andersson B, 1994. Sexual Selection: Princeton University Press.

Arnold SJ, Wade MJ, 1984. On the measurement of natural and sexual selection: applications. Evolution:720-734.

Bailey PC, 1986. The Feeding Behaviour of a Sit-and-Wait Predator, *Ranatra dispar* (Heteroptera: Nepidae): The Combined Effect of Food Deprivation and Prey Size on the Behavioural Components of Prey Capture. Ethology 71:315-332.

Berger D, Walters R, Gotthard K, 2006. What keeps insects small?—Size dependent predation on two species of butterfly larvae. Evol Ecol 20:575-589.

Blest AD, Ocarroll DC, Carter M, 1990. Comparative ultrastructure of layer-I receptor mosaics in principal eyes of jumping spiders - The evolution of regular arrays of light guides. Cell and Tissue Research 262:445-460.

Bond AB, Kamil AC, 2002. Visual predators select for crypticity and polymorphism in virtual prey. Nature 415:609-613.

Briscoe AD, Chittka L, 2001. The evolution of color vision in insects. Annual review of entomology 46:471-510.

Brönmark C, Miner JG, 1992. Predator-Induced Phenotypical Change in Body Morphology in Crucian Carp. Science 258:1348-1350.

Brown PE, Anderson M, 1996. Spectral sensitivity of the compound eye of the cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae). Bulletin of Entomological Research 86:337-342.

Chen X, Dickman CR, Thompson MB, 2004. Selective consumption by predators of different body regions of prey: is rate of energy intake important? Journal of Zoology 264:189-196.

Chevin L-M, Lande R, 2013. Evolution of Discrete Phenotypes from Continuous Norms of Reaction. The American Naturalist 182:13-27.

Chittka L, Waser NM, 1997. Why red flowers are not invisible to bees. Israel Journal of Plant Sciences 45:169-183.

Churchfield S, Hollier J, Brown V, 1991. The effects of small mammal predators on grassland invertebrates, investigated by field exclosure experiment. Oikos:283-290.

Cogni R, Freitas A, Amaral Filho B, 2002. Influence of prey size on predation success by *Zelus longipes* L.(Het., Reduviidae). Journal of Applied Entomology 126:74-78.

Cook L, Saccheri I, 2013. The peppered moth and industrial melanism: evolution of a natural selection case study. Heredity 110:207-212.

Curio E, 2012. The ethology of predation: Springer Science & Business Media.

Dayton GH, Saenz D, Baum KA, Langerhans RB, DeWitt TJ, 2005. Body shape, burst speed and escape behavior of larval anurans. Oikos 111:582-591.

Dixon A, 1959. An experimental study of the searching behaviour of the predatory coccinellid beetle *Adalia decempunctata* (L.). The Journal of Animal Ecology:259-281.

Dixon A, Hemptinne J-L, 2001. Body size distribution in predatory ladybird beetles reflects that of their prey. Ecology 82:1847-1856.

Dixon A, Russel R, 1972. The effectiveness of *Anthocoris nemorum* and *A. confusus* as predators of the sycamore aphid *Drepanosiphum platanoides*. II. Searching behaviour and the incidence of predation in the field. Entomol exp appl 15:35-50.

Domenici P, Turesson H, Brodersen J, Brönmark C, 2008. Predator-induced morphology enhances escape locomotion in crucian carp. Proceedings of the Royal Society B: Biological Sciences 275:195-201.

Dyer LA, 1997. Effectiveness of caterpillar defenses against three species of invertebrate predators. Journal of Research on the Lepidoptera 34:48-68.

Dyer LA, Floyd T, 1993. Determinants of predation on phytophagous insects: the importance of diet breadth. Oecologia 96:575-582.

Edmunds M, 1974. Defence in animals: a survey of anti-predator defences. Longman Publishing Group.

Emlen DJ, 1996. Artificial selection on horn length-body size allometry in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). Evolution:1219-1230.

Emlen DJ, 1997. Alternative reproductive tactics and male-dimorphism in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). Behavioral Ecology and Sociobiology 41:335-341.

Fabricant SA, Herberstein ME, 2015. Hidden in plain orange: aposematic coloration is cryptic to a colorblind insect predator. Behavioral Ecology 26:38-44.

Fagan WF, Hurd L, 1994. Hatch density variation of a generalist arthropod predator: population consequenes and community impact. Ecology:2022-2032.

Feltmate BW, Williams DD, 1989. A test of crypsis and predator avoidance in the stonefly *Paragnetina media* (Plecoptera, Perlidae). Animal Behaviour 37:992-999.

Freitak D, Vanatoa A, Ots I, Rantala MJ, 2005. Formation of melanin-based wing patterns is influenced by condition and immune challenge in *Pieris brassicae*. Entomologia experimentalis et applicata 116:237-243.

Gaston KJ, Chown S, Styles C, 1997. Changing size and changing enemies: the case of the mopane worm. Acta Oecologica 18:21-26.

Goerlitz H, Siemers BM, 2007. Sensory ecology of prey rustling sounds: acoustical features and their classification by wild grey mouse lemurs. Functional Ecology 21:143-153.

Goldsmith TH, 1965. Do Flies Have A Red Receptor? The Journal of General Physiology 49:265-287.

Gottschalk PG, Dunn JR, 2005. The five-parameter logistic: A characterization and comparison with the four-parameter logistic. Analytical Biochemistry 343:54-65.

Griffiths D, 1980. Foraging costs and relative prey size. The American Naturalist 116:743-752.

Guilford T, 1990. The evolution of aposematism. Insect defenses: adaptive mechanisms and strategies of prey and predators:23-61.

Hargeby A, Johansson J, Ahnesjö J, 2004. Habitat-specific pigmentation in a freshwater isopod: adaptive evolution over a small spatiotemporal scale. Evolution 58:81-94.

Harland DP, Jackson RR, 2000. 'Eight-legged cats' and how they see - a review of recent research on jumping spiders (Araneae: Salticidae). Cimbebasia 16:231-240.

Hooper RE, Tsubaki Y, Siva-Jothy MT, 1999. Expression

of a costly, plastic secondary sexual trait is correlated with age and condition in a damselfly with two male morphs. Physiological Entomology 24:364-369.

Horridge GA, Mimura K, Tsukahara Y, 1975. Fly Photoreceptors. II. Spectral and Polarized Light Sensitivity in the Drone Fly *Eristalis*. Proceedings of the Royal Society of London B: Biological Sciences 190:225-237.

Houde AE, 1994. Effect of artificial selection on male colour patterns on mating preference of female guppies. Proceedings of the Royal Society of London B: Biological Sciences 256:125-130.

Iwasaki T, 1991. Predatory behavior of the praying mantis, *Tenodera aridifolia* II. Combined effect of prey size and predator size on the prey recognition. Journal of ethology 9:77-81.

Jackson RR, Li DQ, 2004. One-encounter search-image formation by araneophagic spiders. Animal Cognition 7:247-254.

Johannesson K, Ekendahl A, 2002. Selective predation favouring cryptic individuals of marine snails (*Littorina*). Biological Journal of the Linnean Society 76:137-144.

Jones MP, Pierce KE, Ward D, 2007. Avian vision: a review of form and function with special consideration to birds of prey. Journal of Exotic Pet Medicine 16:69-87.

Kristensen CO, 1994. Investigations on the natural mortality of eggs and larvae of the large white *Pieris brassicae* (L.) (Lep., Pieridae). Journal of Applied Entomology 117:92-98.

Land MF, 1969a. Movements of retinae of jumping spiders (Salticidae - Dendryphantinae) in response to visual stimuli. Journal of Experimental Biology 51:471-493.

Land MF, 1969b. Structure of retinae of principal eyes of jumping spiders (Salticidae - Dendryphantinae) in relation to visual optics. Journal of Experimental Biology 51:443-470.

Land MF, 1971. Orientation by jumping spiders in absence of visual feedback. Journal of Experimental Biology 54:119-&.

Land MF, 1972. Mechanisms of orientation and pattern recognition by jumping spiders (Salticidae). Information Processing in the Visual Systems of Anthropods Berlin Heidelberg: Springer. p. 231-247.

Land MF, 1974. A comparison of the visual behavior of a predatory arthropod with that of a mammal. Invertebrate neurons and behavior MIT Press, Cambridge:411-418.

Land MF, 1985. Fields of view of the eyes of primitive jumping spiders. Journal of Experimental Biology 119:381-384.

Land MF, 1997a. The resolution of insect compound eyes. Israel Journal of Plant Sciences 45:79-91.

Land MF, 1997b. Visual acuity in insects. Annual review of entomology 42:147-177.

Land MF, Nilsson D-E, 2012. Animal eyes: Oxford University Press.

Lande R, Arnold SJ, 1983. The Measurement of Selection on Correlated Characters. Evolution 37:1210-1226.

Lang A, Filser J, Henschel JR, 1999. Predation by ground beetles and wolf spiders on herbivorous insects in a maize crop. Agriculture, ecosystems & environment 72:189-199.

Langerhans RB, 2009. Morphology, performance, fitness: functional insight into a post-Pleistocene radiation of mosquitofish. Biology Letters 5:488-491.

Lattin JD, 1989. Bionomics of the Nabidae. Annual review of entomology 34:383-400.

Loiselle BA, Farji-Brener AG, 2002. What's Up? An Experimental Comparison of Predation Levels between Canopy and Understory in a Tropical Wet Forest. Biotropica 34:327-330.

Lundvall D, Svanbäck R, Persson L, Byström P, 1999. Size-dependent predation in piscivores: interactions between predator foraging and prey avoidance abilities. Canadian Journal of Fisheries and Aquatic Sciences 56:1285-1292.

Maiorana VC, 1981. Prey selection by sight: random or economic? The American Naturalist 118:450-451.

Maly EJ, 1970. The influence of predation on the adult sex ratios of two copepod species. Limnol Oceanogr 15:566-573.

Mänd T, Tammaru T, Mappes J, 2007. Size dependent predation risk in cryptic and conspicuous insects. Evol Ecol 21:485-498.

Marples NM, Kelly DJ, Thomas RJ, 2005. Perspective: the evolution of warning coloration is not paradoxical. Evolution 59:933-940.

Masta SE, Maddison WP, 2002. Sexual selection driving diversification in jumping spiders. Proceedings of the National Academy of Sciences of the United States of America 99:4442-4447.

Mattson WJ, Knight FB, Allen DC, Foltz JL, 1968. Vertebrate predation on the jack-pine budworm in Michigan. Journal of Economic Entomology 61:229-234.

McCann GD, Arnett DW, 1972. Spectral and Polarization Sensitivity of the Dipteran Visual System. The Journal of General Physiology 59:534-558.

McCracken D, Bignal E, Blake S, Foster G, 2004. Productivity and profitability: the effects of farming practices on the prey of insectivorous birds. Insect and Bird Interactions Intercept, Andover, UK:75-87.

Meyer-Rochow VB, Eguchi E, 1984. Thoughts on the possible function and origin of bioluminescence in the New Zealand glowworm *Arachnocampa luminosa* (Diptera: Keroplatidae), based on electrophysiological recordings of spectral responses from the eyes of male adults. New Zealand Entomologist 8:111-119.

Mikolajewski DJ, Johansson F, Wohlfahrt B, Stoks R, 2006. Invertebrate predation selects for the loss of a morphological antipredator trait. Evolution 60:1306-1310.

Moczek AP, Hunt J, Emlen DJ, Simmons LW, 2002. Threshold evolution in exotic populations of a polyphenic beetle. Evolutionary Ecology Research 4:587.

Morin PJ, 1984. The impact of fish exclusion on the abundance and species composition of larval odonates: results of short-term experiments in a North Carolina farm pond. Ecology:53-60.

Morris R, 1963. The effect of predator age and prey defense on the functional response of *Podisus maculiventris* Say to the density of *Hyphantria cunea* Drury. The Canadian Entomologist 95:1009-1020.

Muir LE, Thorne MJ, Kay BH, 1992. *Aedes aegypti* (Diptera: Culicidae) Vision: Spectral Sensitivity and Other Perceptual Parameters of the Female Eye. Journal of Medical Entomology 29:278-281.

Nentwig W, Blick T, Gloor D, Hänggi A, Kropf C, 2016. Spiders of Europe.

New TR, 1992. Insects as predators: New South Wales Univerity Press in association with Australian Institute of Biology.

Nilsson M, Forsman A, 2003. Evolution of conspicuous colouration, body size and gregariousness: a comparative analysis of lepidopteran larvae. Evol Ecol 17:51-66.

Norberg RÅ, 1978. Energy Content of Some Spiders and Insects on Branches of Spruce (*Picea abies*) in Winter; Prey of Certain Passerine Birds. Oikos 31:222-229.

O'Brien WJ, Slade NA, Vinyard GL, 1976. Apparent size as the determinant of prey selection by bluegill sunfish (*Lepomis macrochirus*). Ecology:1304-1310.

Okuyama T, 2007. Prey of two species of jumping spiders in the field. Applied entomology and zoology 42:663-668.

Osorio D, Vorobyev M, 2008. A review of the evolution of animal colour vision and visual communication signals. Vision Research 48:2042-2051.

Ovadia O, Schmitz OJ, 2002. Linking individuals with ecosystems: experimentally identifying the relevant organizational scale for predicting trophic abundances. Proceedings of the National Academy of Sciences 99:12927-12931.

Parker A, 1965. The predatory behaviour and life history of *Pisilus tipuliformis* Fabricius (Hemiptera: Reduviidae). Entomologia Experimentalis et Applicata 8:1-12.

Parker A, 1969. The predatory and reproductive behaviour of *Rhinocoris bicolor* and *R. tropicus* (Hemiptera: Reduviidae). Entomologia Experimentalis et Applicata 12:107-117.

Parker GA, 1972. Reproductive Behaviour of Sepsis cynipsea (L.) (Diptera: Sepsidae). I. A Preliminary Analysis of the Reproductive Strategy and Its Associated Behaviour Patterns. Behaviour 41:172-206.

Partridge L, 1987. Life history constraints on sexual selection. In: Bradbury J, Andersson M, editors. Sexual Selection: Testing the Alternatives London, UK: John Wiley.

Pickens LG, 1990. Colorimetric versus Behavioral Studies of Face Fly (Diptera: Muscidae) Vision. Environmental Entomology 19:1242-1252.

Pont AC, Meier R, 2002. The Sepsidae (Diptera) of Europe [electronic resource]: Brill.

Poulton EB, 1890. The colours of animals: their meaning and use, especially considered in the case of insects: D. Appleton.

Prop N, 1960. Protection against birds and parasites in some species of tenthredinid larvae. Archives Néerlandaises de Zoologie 13:380-447.

Author. 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Reichle D, 1968. Relation of Body Size to Food Intake, Oxygen Consumption, and Trace Element Metabolism in Forest Floor Arthropods. Ecology 49:538-542.

Remmel T, Davison J, Tammaru T, 2011. Quantifying predation on folivorous insect larvae: the perspective of life-history evolution. Biological Journal of the Linnean Society 104:1-18.

Remmel T, Tammaru T, 2009. Size-dependent predation risk in tree-feeding insects with different colouration strategies: a field experiment. Journal of Animal Ecology 78:973-980.

Ruxton GD, Sherratt TN, Speed MP, 2004. Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals and Mimicry. Oxford: Oxford University Press.

Schmitt R, Holbrook S, 1984. Gape-limitation, foraging tactics and prey size selectivity of two microcarnivorous species of fish. Oecologia 63:6-12.

Schülert N, Dicke U, 2002. The effect of stimulus features on the visual orienting behaviour of the salamander *Plethodon jordani*. Journal of experimental biology 205:241-251.

Schwind R, Schlecht P, Langer H, 1984. Microspectrophotometric characterization and localization of three visual pigments in the compound eye of *Notonecta glauca* L. (Heteroptera). J Comp Physiol 154:341-346.

Shine R, 1991. Why do Larger Snakes Eat Larger Prey Items? Functional Ecology 5:493-502.

Shine R, Thomas J, 2005. Do lizards and snakes really differ in their ability to take large prey? A study of relative prey mass and feeding tactics in lizards. Oecologia 144:492-498.

Smola U, Meffert P, 1979. The spectral sensitivity of the visual cells R7 and R8 in the eye of the blowfly *Calliphora erythrocephala*. Journal of comparative physiology 133:41-52.

Stark WS, Walker JA, Harris WA, 1976. Genetic dissection of the photoreceptor system in the compound eye of *Drosophila melanogaster*. The Journal of Physiology 256:415-439.

Stephens DW, Krebs JR, 1986. Foraging theory: Princeton University Press.

Stevens M, 2007. Predator perception and the interrelation between different forms of protective coloration. Proceedings of the Royal Society of London B: Biological Sciences 274:1457-1464.

Stortenbeker CW, 1967. Observations on the population dynamics of the red locust, *Nomadacris septemfasciata* (Serville), in its outbreak areas. Agric Res Rep Wageningen.

Svanbäck R, Eklöv P, 2011. Catch me if you can—predation affects divergence in a polyphenic species. Evolution 65:3515-3526.

Taborsky M, Brockmann HJ, 2010. Alternative reproductive tactics and life history phenotypes. In: Kappeler P, editor. Animal Behaviour: Evolution and Mechanisms: Springer Berlin Heidelberg. p. 537-586.

Talloen W, Dyck HV, Lens L, 2004. The cost of melanization: Butterfly wing coloration underenvironmental stress. Evolution 58:360-366.

Tarsitano M, Jackson RR, 1994. Jumping spiders make predatory detours requiring movement away from prey. Behaviour 131:65-73.

Taylor LA, Maier EB, Byrne KJ, Amin Z, Morehouse NI, 2014. Colour use by tiny predators: jumping spiders show colour biases during foraging. Animal Behaviour 90:149-157.

Teuschl Y, Reim C, Blanckenhorn WU, 2010. No size-dependent reproductive costs in male black scavenger flies (*Sepsis cynipsea*). Behavioral Ecology 21:85-90.

Théry M, Gomez D, 2010. Insect colours and visual appearance in the eyes of their predators. Advances in Insect Physiology 38:267-353.

Tinbergen L, 1960. The natural control of insects in pinewoods. I. Factors influencing the intensity of predation by songbirds. Archives Neerlandaises de Zoologie 13: 265-336. Ardea 68:11-30.

Troost TA, Kooi BW, Dieckmann U, 2008. Joint evolution of predator body size and prey-size preference. Evol Ecol 22:771-799.

True JR, 2003. Insect melanism: the molecules matter. Trends in ecology & evolution 18:640-647.

Tuomikoski R, 1952. Uber die Nahrung der Empididen-lmagines (Dipt.) in Finland. Ann Ent Fenn 18:170-181.

Vamosi SM, Schluter D, 2004. Character shifts in the defensive armor of sympatric sticklebacks. Evolution 58:376-385.

Van Buskirk J, Schmidt BR, 2000. Predator-induced phenotypic plasticity in larval newts: trade-offs, selection, and variation in nature. Ecology 81:3009-3028.

Vencl FV, Srygley RB, 2013. Enemy targeting, trade-offs, and the evolutionary assembly of a tortoise beetle defense arsenal. Evol Ecol 27:237-252.

Vermeij GJ, 1982. Unsuccessful predation and evolution. American Naturalist:701-720.

Wheater CP, 1989. Prey detection by some predatory Coleoptera (Carabidae and Staphylinidae). Journal of Zoology 218:171-185.

Whitman DW, Vincent S, 2008. Large size as an antipredator defense in an insect. Journal of

Orthoptera Research 17:353-371.

Williams DS, McIntyre P, 1980. The principal eyes of a jumping spider have a telephoto component. Nature 288:578-580.

Wilson DS, 1975. The adequacy of body size as a niche difference. The American Naturalist 109:769-784.

Winfield IJ, Townsend CR, 1983. The cost of copepod reproduction: increased susceptibility to fish predation. Oecologia 60:406-411.

Wooster D, 1994. Predator impacts on stream benthic prey. Oecologia 99:7-15.

Zurek DB, Cronin TW, Taylor LA, Byrne K, Sullivan MLG, Morehouse NI, 2015. Spectral filtering enables trichromatic vision in colorful jumping spiders. Current Biology 25:R403-R404.

CHAPTER 4

Healthier or bigger? Trade-off mediating male dimorphism in the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae)

Ecological Entomology

Ecological Entomology (2017), DOI: 10.1111/een.12413

Healthier or bigger? Trade-off mediating male dimorphism in the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae)

JUAN P. BUSSO,[1] WOLF U. BLANCKENHORN[1] and DANIEL GONZÁLES-TOKMAN[2] [1]Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zürich, Switzerland and [2]Cátedras CONACYT, Instituto de Ecología A. C., Xalapa, México

Abstract. 1. Life history trade-offs emerge when limited resources are allocated to multiple functions of an organism. Under highly competitive conditions trade-offs can result in alternative phenotypes that differ morphologically and physiologically. Such is the case in insect species that grow under high densities, where competition for resources but also the risk of disease contagion is high, prompting important adjustments in immune response and melanic cuticular pigmentation, with consequent sacrifices in other fitness-related traits.

2. In the present study, the potential trade-offs between total- and active phenoloxidase (PO), body size and body pigmentation in *Sepsis thoracica* black scavenger flies that show alternative male morphs differing in cuticular pigmentation, and body size were evaluated.

3. As expected, small/dark (*obsidian*) males showed higher total-PO activity than larger/orange (*amber*) males. A negative relationship was found between total-PO activity and body size in females and *obsidian* but not *amber* males, suggesting that growth and immunity are more costly for the former. In contrast, density did not affect PO activity, as predicted by the density-dependent prophylaxis hypothesis, which had not been tested in dipterans before. However, rearing density did affect the body size negatively in females and *amber* but not *obsidian* males, showing that male morph is largely determined by condition-dependent plasticity rather than genes.

4. This study provides good evidence that trade-offs between different life-history traits can result in alternative resource allocation strategies, even within one species. These strategies can produce strikingly different alternative phenotypes, evincing that there is not only one optimal solution to address fitness optimisation.

Key words. Body size, coloration, density-dependent prophylaxis, melanin, phenoloxidase, trade-off.

Introduction

The limiting nature of resources requires organisms to carefully distribute their investment into a variety of traits, resulting in life-history trade-offs (Zera & Harshman, 2001). Trade-offs originate because the best phenotype for one task is usually not the best for other tasks (Campbell *et al.,* 1991). Accordingly, organisms tend to maximise their fitness through multi-objective optimisation (Abraham & Jain, 2005; Shoval *et al.,* 2012), which not necessarily results in only one optimal solution (Farnsworth & Niklas, 1995).

Among these trade-offs, the relative resource allocation to body size and immunity is of particular interest because both traits influence fitness in different but complementary ways. Body size provides fitness benefits in almost every aspect of an organism's biology (reviewed by LaBarbera, 1986; Peters, 1986), whereas immunity provides benefits against pathogens, against which body size is no defence. Hence, organisms face the challenge to attain the benefits of larger body size (Kingsolver

Correspondence: Juan P. Busso, Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zürich, Switzerland. E-mail: juanpablo.busso@ieu.uzh.ch

& Pfennig, 2004; Hone & Benton, 2005) while retaining the possibility to defend it against pathogens. This investment in immunity strongly compromises the investment into other traits (Zuk & Stoehr, 2002; Schmid-Hempel, 2003). As maintaining an immune system is very costly (Schmid-Hempel, 2003), and body size is also resource-demanding (Reichle, 1968; Hone & Benton, 2005), we hypothesise that there will be a trade-off between these two traits, as has been observed in some other insects (Rantala & Roff, 2005) but often also not (Suwanchaichinda & Paskewitz, 1998; Vainio *et al.,* 2004; Córdoba-Aguilar *et al.,* 2009). These contradicting results drove us to address this relationship in this study.

The immune system of insects defends the organism against pathogens via humoural and cellular components (Schmid-Hempel, 2005; Rolff & Reynolds, 2009). The humoural immune response targets mainly microbial pathogens (Boman & Hultmark, 1987; Morishima *et al.,* 1995; Adamo, 2004), whereas the cellular response targets primarily foreign intrusions of fungi, nematodes, parasitoids, and mites (Schmid-Hempel, 2005; Siva-Jothy *et al.,* 2005). An important component of the cellular response is the encapsulation of intruders, which results from the phenoloxidase (PO) cascade (Iwanaga & Lee, 2005; González-Santoyo & Córdoba-Aguilar, 2012). This cascade ends in the production of melanin, which is deposited around the invaders in nodules and capsules, resulting in their isolation (Schmid-Hempel, 2005; Strand, 2008). Interestingly, the immunity-related expression of melanin influences the cuticular coloration of insects (Aso *et al.,* 1985; Wilson & Reeson, 1998; Barnes & Siva-Jothy, 2000; Wilson *et al.,* 2001; Armitage & Siva-Jothy, 2005). It is, therefore, further expected that the trade-off between body size and immunity should result in a negative correlation between body size and cuticular melanisation.

Investment in immunity is also a function of the resources acquired, which in turn strongly depends on the competitive environment in terms of population density (Agnew *et al.,* 2002). Density not only affects resource acquisition but also imposes immunological costs by influencing the risk of disease contagion (Goulson & Jenny, 1995; Patterson & Ruckstuhl, 2013; Kappeler *et al.,* 2015). As parasite transmission is often density-dependent, animals living at higher densities are expected to invest more resources into parasite defence, a phenomenon named 'density-dependent prophylaxis' (Wilson & Reeson, 1998) that has found support in various social and non-social arthropods including orthopterans, lepidopterans, thysanopterans, coleopterans, and hymenopterans (Barnes & Siva-Jothy, 2000; Wilson *et al.,* 2002; Cotter *et al.,* 2004b; Stow *et al.,* 2007; Turnbull *et al.,* 2011) but see (Wilson *et al.,* 2003; González-Tokman *et al.,* 2014). Because of the involvement of melanin in insect immunity through the PO cascade, increased pathogen resistance in highly dense groups is often accompanied by cuticular darkening (Wilson *et al.,* 2002; Cotter *et al.*, 2004a). Hence, we expect that the density at which individuals develop will affect the insect's immune response, also altering their coloration.

An excellent system to address the putative trade-off between body size and immunity is the black scavenger fly *Sepsis thoracica* (Robineau-Desvoidy) (Diptera: Sepsidae). Males of this species exhibit extreme variability in size and colour, with smaller males being mostly black and the largest males bright orange, whereas females are completely black (Pont & Meier, 2002). This variation suggests a putative trade-off between size and immunity in the males but not the females. Females of this species lay their eggs into (primarily cow) dung pats from which larvae cannot escape during development. Hence these dung pats are like islands that spatiotemporally vary in intra- and interspecific competitor density depending on the number of eggs laid into them. A highly competitive environment is expected to be more costly (Zuk & Stoehr, 2002; Rantala & Roff, 2005). Consequently, in *S. thoracica* the correlation between body size and melanisation, together with the variable intraspecific competition levels during development, presents a unique opportunity to analyse how body size and (here larval) population density influence the insects' investment into immunity. In this study, individuals at different densities were raised and body size and coloration, as well as the PO activity as a proxy of their immune system, were measured (González-Santoyo & Córdoba-Aguilar, 2012).

Material and methods

Sepsis thoracica maintenance and rearing

Four European *S. thoracica* populations from Asturias, Spain (43.3°N, 6.0°E), Petroia, Italy (43.23°N, 12.56°W), Prati di Stronconi, Italy (42.48°N, 12.68°W), and Borgonuovo, Italy (46.33°N, 9.44°W) were sampled. Wild-caught females were brought to the laboratory and used to establish cultures of multiple (10–20) replicate iso-female lines per population that were housed in separate plastic containers and regularly supplied with fresh cow dung, sugar, and water *ad libitum*. The cultures were maintained at 18 ± 1 °C under an LD 14:10 h.

In this study, to homogenise any possible effects of local adaption, we used a random mix of *S. thoracica* flies from at least two lines of each of the populations mentioned above. To raise the flies, a container with dung ($L \times W \times H = 10 \times 20 \times 10$ mm) was placed for 24 h inside any of the *S. thoracica* stock cultures for females to deposit eggs. This dung was incubated afterwards in a novel container to obtain experimental adult flies. For each container, we assessed the density of emerged adults reflecting differences in food limitation owing to larval competition. The foreleg femur area was measured, which is an excellent proxy for both body size and coloration, based on previous analyses. For all *S. thoracica* flies the foreleg femur was photographed under a stereomicroscope MZ12 with a DFC490 camera (Leica, Heerbrugg, Switzerland), against a neutral white background. The camera was calibrated with a mini IT-8 calibration target, to guarantee colour consistency between the different pictures. The target also served as a scale to posteriorly measure size. A self-written code in ImageJ was used, which measured the amount of pixels of the foreleg femur, and converted them to the real area according to the scale correspondent to each picture. The code also measured the amount of melanic pixels in the femur, guaranteeing an objective quantification of melanism. The melanic colour of the flies was defined as any pixel with a V value over 163, in the YUV colorspace. This

 Ecological Entomology, doi: 10.1111/een.12413

threshold was selected, as it corresponded to the valley in the V values distribution present in the femur coloration. Melanism represented the proportion of black and brown pixels over all the pixels of the femur. The phenoloxidase activity from 74 randomly selected individuals of both sexes encompassing the entire body size range was further measured.

Measurement of phenoloxidase activity

Melanisation results from the enzymatic activation of prophenoloxidase (proPO) into Phenoloxidase (PO), which converts phenols to quinones that ultimately polymerise to form melanin (Söderhäll & Cerenius, 1998). PO is the ready-to-use enzyme that has been naturally activated by the insect (henceforth 'active-PO'), but the total investment in the PO pathway includes both proPO and active-PO (henceforth 'total-PO'). Consequently, active-PO refers to the amount of naturally activated enzyme when taking the measurement, whereas total-PO is the overall investment in the immune pathway. Active-PO can be measured directly as described below, but to estimate total-PO, proPO has to be converted into PO using chymotrypsin (González-Santoyo & Córdoba-Aguilar, 2012).

As the amount of haemolymph that can be extracted from a single fly is very limited, phenoloxidase activity was quantified from whole individual homogenates (see a similar procedure in Cornet *et al.,* 2013). Flies were crushed individually in 80 µl of cold phosphate-buffered saline (PBS) buffer. Afterwards, we centrifuged the individuals (5 min, 5 °C, 16 060 g) in a spin column (M1003; Mobicol, Goettingen, Germany) with a 10-µm pore filter (M2210; Mobicol). The clean extract was collected for subsequent analysis. For each individual, we measured the protein content, the active-PO and total-PO using a spectrophotometric assay. As whole individual homogenates were used, the extract contains not only haemolymph but also cuticular PO. Nevertheless, both play an important role in the immune defence of insects (Barnes & Siva-Jothy, 2000; Cotter *et al.*, 2004b; Armitage & Siva-Jothy, 2005). Furthermore, cuticular PO is strongly correlated with haemolymph PO because the precursor proPO resides in the hemocytes (Hoffmann, 1995).

To measure proteins, the bicinchoninic acid assay (BCA) was used, for which 5 µl of fly extract was added to a microplate well containing 45 µl cold PBS. Afterwards, 150 µl of BCA solution was added (BCA Protein Assay Kit 23225; Thermo Scientific, Rockford, Illinois). After 9 min, we measured the absorbance at 590 nm and obtained the amount of protein relative to the albumin standard of the same kit.

The rest of the extract was divided into two parts, one to measure total-PO and the other to measure active-PO. To measure total-PO, 20 µl of fly extract was added to a microplate well containing 7 µl of α-chemotrypsin (Sigma C7762, 2 mg ml^{-1} of ddH_2O) and left 10 min at room temperature to convert proPO into PO. Next, 20 µl of cold PBS and 133 µl of ddH_2O were added. Lastly, 10 µl of L-Dopa solution (the substrate for the PO, Sigma D9628, 2 mg ml^{-1} of ddH_2O) was added to the mix and the colorimetric reaction was followed in a microplate reader (SpectraMax 340PC384; Molecular Devices, Sunnyvale, California) at 30 °C. Readings were taken for 4 h at 490 nm.

To measure active-PO, we added to 40 µl of fly extract 10 µl of protease inhibitor (Sigma P2714), to avoid endogenous enzymes from activating more proPO. Afterwards, 20 µl of this mix was added to a microplate well containing 20 µl of cold PBS, and then 140 µl of ddH_2O was added. After that, 10 µl of L-Dopa solution was added to the mix, and the colorimetric reaction was followed in a microplate reader as described above. Controls in both essays followed the same procedures, but using cold PBS instead of fly extract (see similar procedures in Cotter *et al.*, 2004a; González-Santoyo *et al.,* 2010).

Statistical analyses

To analyse enzyme activity, we fitted a five-parameter logistic regression model (Gottschalk & Dunn, 2005) to the readings of the colorimetric reaction. The slope (Vmax) of the reaction was measured during the linear phase (inflection point). To control for the autonomous conversion of L-Dopa to dopaquinone we subtracted the activity of the control essays. These values were then divided by the protein amount to control for extract concentration differences. The reported values refer to activity present per micrograms of protein.

Males were subdivided into two groups based on the bimodal distribution observed for their coloration: obsidians (black and small) and amber (orange and large). The two male morphs plus the females composed the three levels of the factor *morph* in the model. Intermediate phenotypes were removed from the analyses owing to their ambiguity and low number. Hence the sample size for all analyses is $N = 68$.

To analyse the relation between body size and coloration in *S. thoracica* we fitted a five-Parameter logistic regression model: Orangeness = $c + (d\text{-}c)/[1 + (BS/e)\text{^}b]\text{^}f$, where BS is body size, d is the estimated coloration at body size zero, c is the estimated Orangeness at infinite body size, e is the mid-range body size, b is the slope factor, and f is the asymmetry factor (Gottschalk & Dunn, 2005). This function is an improvement from the four-Parameter logistic regression improving accuracy for even light asymmetries in the logistic curve.

To determine the effect of larval density on body size, we fitted a generalised mixed effect model. The model included the factor morph, larval density as a continuous covariate, and the possible two-way interaction between these two. We also incorporated in the model a random effect of line nested within populations, to control for their relatedness. The significance of the differences in body size between morphs was evaluated with the Tukey's 'Honest Significant Difference' method.

To analyse the influence of body size, density and morph on the immune system, we also employed generalised mixed effect models in the lme4 package. The model included the predictor's morph, body size and density, plus all the possible two-way interactions between these variables as well as the three-way interaction. Also incorporated in the model was a random effect of line nested within populations. The same model for active, and total-PO was used. Significance of the predictor variables was calculated with likelihood ratio tests. The significance of the differences in total-PO between morphs was evaluated with the Tukey's 'Honest Significant Difference' method. All analyses

 Ecological Entomology, doi: 10.1111/een.12413

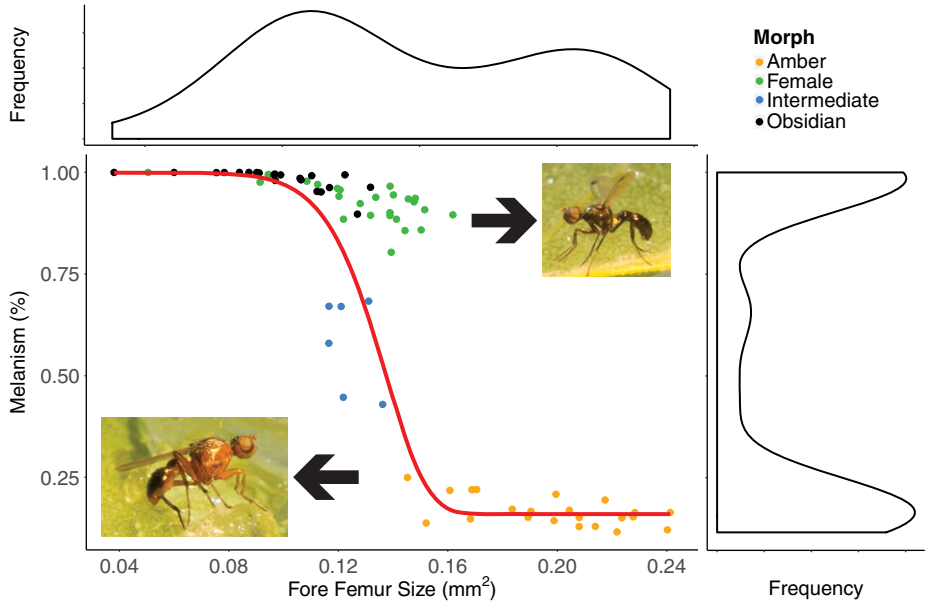


Fig. 1. Relationship between body size and melanisation in *Sepsis thoracica*, and their respective frequency distributions for males. The pictures show the amber and obsidian male morphs. [Colour figure can be viewed at wileyonlinelibrary.com].

were done using the software R Version 3.2.2 (R Development Core Team, 2015).

Results

A sigmoid relationship between body size and coloration in *S. thoracica* males across all the sampled populations was found, featuring bimodal body size and bimodal coloration distributions (Fig. 1). The five-parameter non-linear regression model of these two variables fitted well this relationship (Fig. 1, $R^2 = 0.924$). Female coloration overlaps with that of the obsidian males, whereas their body size is intermediate between the two male morphs (Fig. 2a).

Body size differed between morphs (Fig. 2, $\chi^2 = 205.309$, d.f. = 2, $P < 0.001$), and also decreased with larval density overall ($\chi^2 = 21.918$, d.f. = 1, $P < 0.001$). However, the interaction between these two variables was also significant ($\chi^2 = 12.872$, d.f. = 2, $P = 0.002$), owing to the relatively flat slope of the obsidian males relative to females and amber males (Fig. 3).

Active-PO did not vary between morphs ($\chi^2 = 4.169$, d.f. = 2, $P = 0.244$; females = 1.413 ± 0.750, ambers = 1.050 ± 0.402, obsidians = 1.106 ± 0.483; all values $\times e^{-6}$); it was also not affected by any other predictor variable (*body size*, $\chi^2 = 0.037$, d.f. = 1, $P = 0.848$; *density*, $\chi^2 = 0.377$, d.f. = 1, $P = 0.539$) or their two-way (*morph:body-size*, $\chi^2 = 3.619$, d.f. = 2, $P = 0.306$; *morph:density*, $\chi^2 = 1.024$, d.f. = 2, $P = 0.795$; *body-size:density*, $\chi^2 = 1.084$, d.f. = 1, $P = 0.298$) or three-way interactions ($\chi^2 = 2.838$, d.f. = 2, $P = 0.417$).

In contrast, total-PO was significantly influenced by morph, being highest for obsidian males (Fig. 2b, $\chi^2 = 11.860$, d.f. = 2, $P = 0.008$), and decreased with body size overall ($\chi^2 = 15.729$, d.f. = 1, $P < 0.001$), although there was also an interaction in that total-PO was largely unaffected by the body size of the largest, amber males (Fig. 4, $\chi^2 = 14.803$, d.f. = 2, $P = 0.002$). All other effects were non-significant (density, $\chi^2 = 0.005$, d.f. = 1, $P = 0.943$; *morph:density*, $\chi^2 = 0.031$, d.f. = 2, $P = 0.999$; *body-size:density*, $\chi^2 = 1.291$, d.f. = 1, $P = 0.256$; *morph:body size:density*, $\chi^2 = 0.322$, d.f. = 2, $P = 0.956$).

Discussion

Sepsis thoracica males display a sigmoid relationship between body size and coloration, resulting in a body size threshold that divides males into two colour morphs: the small and black morph, which we call obsidian, and the large and orange morph, which we call amber (Fig. 1). These two morphs differ in their total immune investment (total-PO), but not in their naturally activated immune responses (active-PO). Importantly, body size, which is inversely related to rearing density, influences the total immune investment in males and females, revealing a trade-off between the two traits in both sexes, but also across the morphs in that obsidian males focus their investment into immunity while amber males invest more in body size.

Life-history costs often allow for variation between individuals, even within the same sex, in the allocation of resources to different traits (Zuk & Stoehr, 2002). In *S. thoracica*, differential allocation revealed a trade-off between immunity and body size in both males and females. In males, this trade-off is further apparent between morphs, resulting in two strategies: obsidians invest more in immunity and ambers more in body size (Figs 2 and 4). This trade-off between body size and immunity has been observed in other insects (Yan *et al.,* 1997; Rantala & Roff, 2005; Cotter *et al.,* 2008), as well as in vertebrates (Leach's Storm-Petrel: Mauck *et al.,* 2005; Atlantic

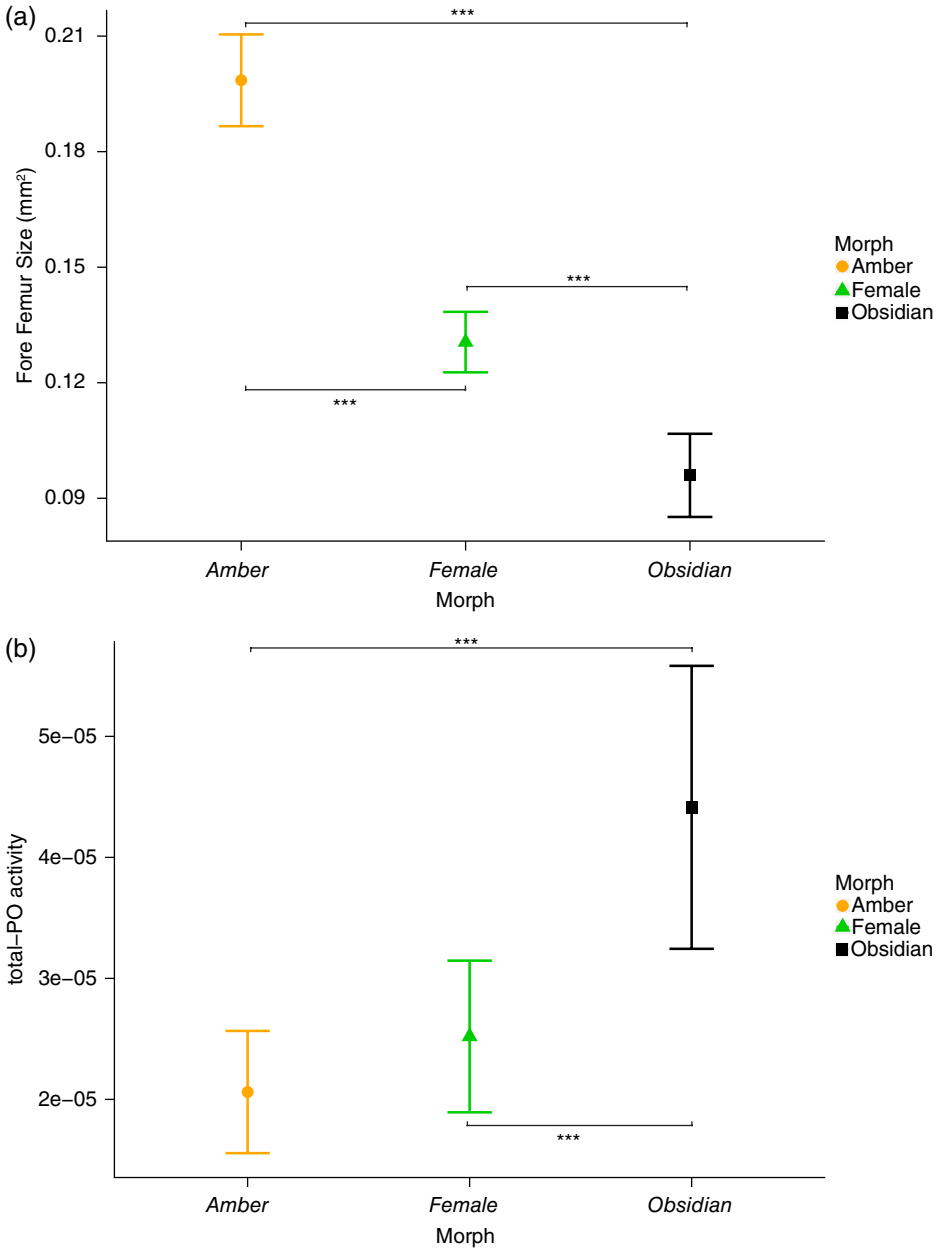


Fig. 2. Differences between females and the two male morphs in body size (a) and total-PO (b). The activity was measured as Absorbance units/min/µg of protein. ***$P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

salmon: Visse *et al.,* 2015). Interestingly, we observed a limit to this trade-off in amber males at the lowest immune investment: their body size keeps increasing whereas their immune investment does not decrease any further (Fig. 4). It is likely that this further body size increase in ambers involves costs other than immunity. Indeed, larger individuals take longer to develop (J. P. Busso, unpublished; Blanckenhorn *et al.,* 2007), which likely entails viability costs (Blanckenhorn, 2000). Nevertheless, the costs of reducing their immunity may be outweighed by the benefits of this larger body size, for example, by decreasing their vulnerability to predators (J. P. Busso, unpublished), or by conferring to the amber males a higher mating success (J. P. Busso, unpublished). Consequently, *S. thoracica* male dimorphism reveals both resource (in terms of energy and nutrients) and viability costs of the immune system (Zuk & Stoehr, 2002), and by choosing either cost, each morph has adopted an alternative solution.

This body size / immunity trade-off concurs with the application of the Bateman's principle to immunity (Rolff, 2002). Originally this hypothesis states that females invest more in immunity to increase their fitness through longevity, whereas males invest less to increase their fitness by augmenting mating

 Ecological Entomology, doi: 10.1111/een.12413

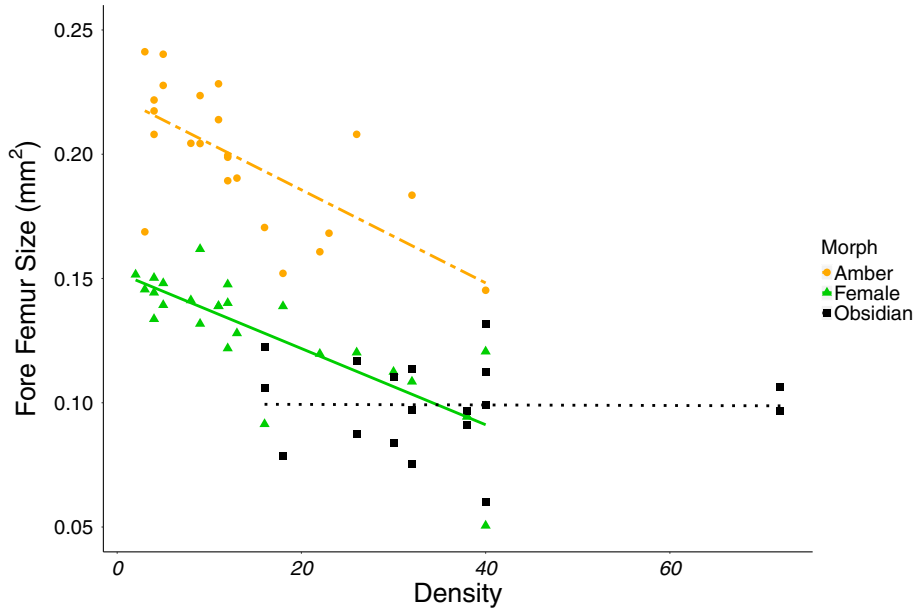


Fig. 3. Effect of larval density on the body size of both sexes and male morphs. [Colour figure can be viewed at wileyonlinelibrary.com].

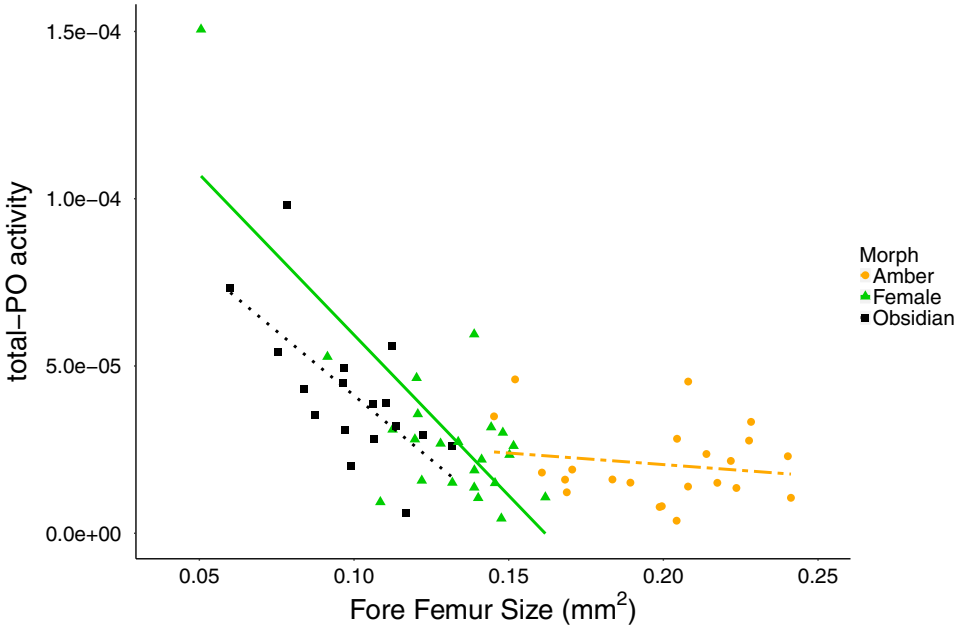


Fig. 4. Effect of body size in total-phenoloxidase (PO) activity in females and both male morphs. The activity was measured as absorbance units/min/µg of protein. [Colour figure can be viewed at wileyonlinelibrary.com].

success. Here we apply this hypothesis not to male / female differences, as seen in some insects (Nunn *et al.,* 2009), but to explain differences between ambers and obsidians. The two *S. thoracica* male morphs provide an excellent opportunity for an intrasexual evaluation of this hypothesis, excluding possible confounding influences of sexually related hormones that also regulate immunity and body coloration in animals (Folstad & Karter, 1992). According to the logic of Rolff's (2002) hypothesis, obsidians would invest more in immunity to increase their fitness through longevity, whereas ambers would invest more in body size and increase their fitness through improved mating success, resulting in alternative reproductive tactics (J. P. Busso, unpublished). A similar situation presents itself for the damselflies *Paraphlebia zoe* Hagen and *Mnais pruinosa* Selys, whose two male morphs also employ alternative reproductive tactics that correlate with different immune investments (Tsubaki *et al.,* 1997; Tsubaki & Hooper, 2004; Ruiz-Guzmán *et al.,* 2013). Hence, species with alternative reproductive tactics

suggest that the Bateman's principle can explain not only differential immune investment between but also within the sexes.

Alternatively, the different immune investments between morphs could result from differences in their rearing densities (Svensson *et al.,* 2001; Wilson & Cotter, 2008). The 'density-dependent prophylaxis' hypothesis (Wilson & Reeson, 1998) predicts that individuals that live at higher densities should invest more in immunity in preparing for a more stressful larval and adult environment. However, the present results indicate that larval density does not directly affect investment into immunity because density negatively influenced the body size of ambers and females but not obsidians (Fig. 3). It is conceivable that increased competition at higher larval densities influences the resources individuals invest in body size and immunity, although the lacking density effect on the obsidians' body size does not support this hypothesis. However, owing to the difficulty of directly manipulating and assessing larval density in the dung without damaging the larvae or affecting their immune system, the number of emerged adults was used as a proxy. This indirect assessment of larval competition could have masked other potentially confounding effects, such as e.g. larval mortality along the way. Unfortunately, it is difficult to evaluate the extent of the indirect density effect (through body size) in the context of the density-dependent prophylaxis hypothesis as most other studies did not assess the body size of individuals (Barnes & Siva-Jothy, 2000; Wilson *et al.,* 2003; González-Tokman *et al.,* 2014). Future studies should evaluate the effect of density on other immune components, such as the resulting melanic encapsulation response or antibacterial lysozyme activity, which are negatively affected by rearing density in other insects (Barnes & Siva-Jothy, 2000; Cotter *et al.*, 2004a).

Nevertheless, the higher total investment in immunity by the black obsidian morph concurs with the correlation between immunity and cuticular pigmentation observed in several insect taxa (Mitsui & Kunimi, 1988; Kunimi & Yamada, 1990; Reeson *et al.,* 1998; Cotter *et al.*, 2004b; Armitage & Siva-Jothy, 2005). Based on this evidence one could argue that the melanic coloration could be an honest signal to parasitoids of an immunocompetent host that has high PO activity and should consequently be avoided (Wilson *et al.,* 2001). However, the fact that female *S. thoracica* are as black as the obsidians and have a total-PO activity similar to the ambers does rule out a simple relationship between PO and melanic coloration here. In fact, there are several other species that do not show higher PO activity of melanic phenotypes (Barnes & Siva-Jothy, 2000; Wilson *et al.,* 2002). Therefore, more evidence is needed to assess the influence and significance of immunity on cuticular coloration. In *S. thoracica*, if the cuticular melanisation acts as an immunocomptence signal, it would only do so in the males. As we found both male morphs in all isofemale lines of all populations, we conclude that male coloration is not determined genetically but rather by condition-dependent plasticity, and intermediate phenotypes are rare but exist owing to the sigmoid shape of the body size–melanism relationship.

In contrast, the lack of differences for active-PO, which constitutes between 3% and 6% of total-PO, indicates that all individuals show similar investment into their active immune system when lacking a pathogenic threat. These differences between the total immune investment and active immunity are because of high costs of deploying an immune response (Schmid-Hempel, 2003; Verhulst *et al.,* 2005). Mounting an immune response involves resource costs to synthesise and maintain the PO-cascade (González-Santoyo & Córdoba-Aguilar, 2012), as well as autoreactive immune costs of the cytotoxic by-products of this cascade (Schmid-Hempel, 2003; González-Santoyo & Córdoba-Aguilar, 2012). Consequently, individuals should minimise the activation of their immune system in the absence of pathogens, resulting in no obvious differences in their active immune system, as observed in our study. However, to provide a full immune picture, future studies are needed to analyse the humoral response in each morph. Complementary immune measurements will surely help interpret the immune investments, but it might not be easy to assess their relative impact on the individual's fitness, owing to the complexity of the immune system (Schmid-Hempel, 2005; Strand, 2008).

To conclude, we found in *S. thoracica* two male morphs defined by their coloration and body size that also show differential investment in their immunity. This differential investment results from a trade-off between immunity and body size, which is also observed in the females. Besides the costs of immunity in terms of reduced body size, the amber morphs suggest further indirect viability costs of immunity owing to the longer development necessary to gather more resources to express larger body size (Blanckenhorn *et al.,* 2007). The coloration of the amber morph seems to result from a drastic drop in investment into immunity. Hence it is possible that melanic coloration acts as a signal of the immunocompetence of males. To our knowledge, this is the first study evaluating density-dependent prophylaxis in dipterans (Wilson & Reeson, 1998), although the lacking effect of larval density on both active and total-PO does not support this hypothesis. Nevertheless, *S. thoracica* is to our knowledge the first example of a trade-off between body size and immunity that strongly divides the morphospace of the males into two strikingly different morphs: ambers and obsidians. This male dimorphism supports the idea that there is not only one optimal solution to address fitness optimisation (Farnsworth & Niklas, 1995).

Acknowledgements

We thank the people at the Castello di Petroia, for letting us collect fly specimens on their fields. We would also like to thank the Blanckenhorn Group (Patrick, Martin, Athene, Valerian, Stefan) for the commentaries and recommendations when analysing the data.

References

Abraham, A. & Jain, L. (2005) Evolutionary Multiobjective Optimization. *Evolutionary Multiobjective Optimization: Theoretical Advances and Applications* (ed. by A. Abraham, L. Jain and R. Goldberg), pp. 1–6. Springer, London, U.K.

Adamo, S.A. (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*, **50**, 209–216.

Agnew, P., Hide, M., Sidobre, C. & Michalakis, Y. (2002) A minimalist approach to the effects of density-dependent competition on insect life-history traits. *Ecological Entomology*, **27**, 396–402.

Armitage, S.A.O. & Siva-Jothy, M.T. (2005) Immune function responds to selection for cuticular colour in *Tenebrio molitor*. *Heredity*, **94**, 650–656.

Aso, Y., Kramer, K.J., Hopkins, T.L. & Lookhart, G.L. (1985) Characterization of haemolymph protyrosinase and a cuticular activator from *Manduca sexta* (L.). *Insect Biochemistry*, **15**, 9–17.

Barnes, A.I. & Siva-Jothy, M.T. (2000) Density–dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **267**, 177–182.

Blanckenhorn, W.U. (2000) The evolution of body size: what keeps organisms small? *Quarterly Review of Biology*, **75**, 385–407.

Blanckenhorn, W.U., Meier, R. & Teder, T. (2007) Rensch's rule in insects: patterns among and within species. *Sex, Size, and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*, pp. 60–70. Oxford University Press, Oxford, U.K.

Boman, H.G. & Hultmark, D. (1987) Cell-free immunity in insects. *Annual Review of Microbiology*, **41**, 103–126.

Campbell, B., Grime, J. & Mackey, J. (1991) A trade-off between scale and precision in resource foraging. *Oecologia*, **87**, 532–538.

Córdoba-Aguilar, A., Jiménez-Cortés, J.G. & Lanz-Mendoza, H. (2009) Seasonal variation in ornament expression, body size, energetic reserves, immune response, and survival in males of a territorial insect. *Ecological Entomology*, **34**, 228–239.

Cornet, S., Gandon, S. & Rivero, A. (2013) Patterns of phenoloxidase activity in insecticide resistant and susceptible mosquitoes differ between laboratory-selected and wild-caught individuals. *Parasites & Vectors*, **6**, 1–11.

Cotter, S., Hails, R., Cory, J.S. & WIlson, K. (2004a) Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, **73**, 283–293.

Cotter, S.C., Kruuk, L.E.B. & Wilson, K. (2004b) Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology*, **17**, 421–429.

Cotter, S., Myatt, J., Benskin, C. & Wilson, K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology*, **21**, 1744–1754.

Farnsworth, K.D. & Niklas, K.J. (1995) Theories of optimization, form and function in branching architecture in plants. *Functional Ecology*, **9**, 355–363.

Folstad, I. & Karter, A.J. (1992) Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*, **139**, 603–622.

González-Santoyo, I. & Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomologia Experimentalis et Applicata*, **142**, 1–16.

González-Santoyo, I., Córdoba-Aguilar, A., González-Tokman, D. & Lanz-Mendoza, H. (2010) Phenoloxidase activity and melanization do not always covary with sexual trait expression in *Hetaerina damselflies* (Insecta: Calopterygidae). *Behaviour*, **147**, 1285–1307.

González-Tokman, D., Ruch, J., Pulpitel, T. & Ponton, F. (2014) Cuticular antifungals in spiders: density-and condition dependence. *PLoS ONE*, **9**, e91785.

Gottschalk, P.G. & Dunn, J.R. (2005) The five-parameter logistic: a characterization and comparison with the four-parameter logistic. *Analytical Biochemistry*, **343**, 54–65.

Goulson, D. & Jenny, S.C. (1995) Responses of *Mamestra brassicae* (Lepidoptera: Noctuidae) to crowding: interactions with disease resistance, colour phase and growth. *Oecologia*, **104**, 416–423.

Hoffmann, J.A. (1995) Innate immunity of insects. *Current Opinion in Immunology*, **7**, 4–10.

Hone, D.W.E. & Benton, M.J. (2005) The evolution of large size: how does Cope's Rule work? *Trends in Ecology & Evolution*, **20**, 4–6.

Iwanaga, S. & Lee, B.-L. (2005) Recent advances in the innate immunity of invertebrate animals. *BMB Reports*, **38**, 128–150.

Kappeler, P.M., Cremer, S. & Nunn, C.L. (2015) Sociality and health: impacts of sociality on disease susceptibility and transmission in animal and human societies. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, **370**, 20140116.

Kingsolver, J.G. & Pfennig, D.W. (2004) Individual-level selection as a cause of Cope's rule of phyletic size increase. *Evolution*, **58**, 1608–1612.

Kunimi, Y. & Yamada, E. (1990) Relationship of larval phase and susceptibility of the armyworm, Pseudaletia separata Walker (Lepidoptera: Noctuidae) to a nuclear polyhedrosis virus and a granulosis virus. *Applied Entomology and Zoology*, **25**, 289–297.

LaBarbera, M. (1986) The evolution and ecology of body size. *Patterns and Processes in the History of Life: Report of the Dahlem Workshop on Patterns and Processes in the History of Life Berlin 1985, June 16–21*, (eds. by D. M. Raup & D. Jablonski), pp. 69–98. Springer, Berlin, Heidelberg.

Mauck, R., Matson, K., Philipsborn, J. & Ricklefs, R. (2005) Increase in the constitutive innate humoral immune system in Leach's Storm-Petrel (*Oceanodroma leucorhoa*) chicks is negatively correlated with growth rate. *Functional Ecology*, **19**, 1001–1007.

Mitsui, J. & Kunimi, Y. (1988) Effect of larval phase on susceptibility of the armyworm, *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) to an entomogenous deuteromycete, *Nomuraea rileyi*. *Japanese Journal of Applied Entomology and Zoology*, **32**, 129–134.

Morishima, I., Horiba, T., Iketani, M., Nishioka, E. & Yamano, Y. (1995) Parallel induction of cecropin and lysozyme in larvae of the silkworm, *Bombyx mori*. *Developmental & Comparative Immunology*, **19**, 357–363.

Nunn, C.L., Lindenfors, P., Pursall, E.R. & Rolff, J. (2009) On sexual dimorphism in immune function. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, **364**, 61–69.

Patterson, J.E. & Ruckstuhl, K.E. (2013) Parasite infection and host group size: a meta-analytical review. *Parasitology*, **140**, 803–813.

Peters, R.H. (ed.) (1986) *The Ecological Implications of Body Size*, p. 329. Cambridge University Press, New York, New York.

Pont, A.C. & Meier, R. (2002) The Sepsidae (Diptera) of Europe. *Fauna Entomologica Scandinavica*, **37**, 1–221.

Rantala, M.J. & Roff, D.A. (2005) An analysis of trade-offs in immune function, body size and development time in the Mediterranean Field Cricket, *Gryllus bimaculatus*. *Functional Ecology*, **19**, 323–330.

R Development Core Team (2015) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Reeson, A.F., Wilson, K., Gunn, A., Hails, R.S. & Goulson, D. (1998) Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **265**, 1787–1791.

Reichle, D. (1968) Relation of body size to food intake, oxygen consumption, and trace element metabolism in forest floor arthropods. *Ecology*, **49**, 538–542.

Rolff, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **269**, 867–872.

Rolff, J. & Reynolds, S. (2009) *Insect Infection and Immunity: Evolution, Ecology, and Mechanisms*. Oxford University Press, Oxford, U.K.

Ruiz-Guzmán, G., Canales-Lazcano, J., Jiménez-Cortés, J.G. & Contreras-Garduño, J. (2013) Sexual dimorphism in immune response: testing the hypothesis in an insect species with two male morphs. *Insect Science*, **20**, 620–628.

Schmid-Hempel, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **270**, 357–366.

Schmid-Hempel, P. (2005) Evolutionary ecology of insect immune defenses. *Annual Review of Entomology*, **50**, 529–551.

Shoval, O., Sheftel, H., Shinar, G., Hart, Y., Ramote, O., Mayo, A. *et al.* (2012) Evolutionary trade-offs, Pareto optimality, and the geometry of phenotype space. *Science*, **336**, 1157–1160.

Siva-Jothy, M.T., Moret, Y. & Rolff, J. (2005) Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology*, **32**, 1–48.

Söderhäll, K. & Cerenius, L. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, **10**, 23–28.

Stow, A., Briscoe, D., Gillings, M., Holley, M., Smith, S., Leys, R. *et al.* (2007) Antimicrobial defences increase with sociality in bees. *Biology Letters*, **3**, 422–424.

Strand, M.R. (2008) The insect cellular immune response. *Insect Science*, **15**, 1–14.

Suwanchaichinda, C. & Paskewitz, S.M. (1998) Effects of larval nutrition, adult body size, and adult temperature on the ability of *Anopheles gambiae* (Diptera: Culicidae) to melanize Sephadex beads. *Journal of Medical Entomology*, **35**, 157–161.

Svensson, E., Sinervo, B. & Comendant, T. (2001) Density-dependent competition and selection on immune function in genetic lizard morphs. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 12561–12565.

Tsubaki, Y. & Hooper, R.E. (2004) Effects of eugregarine parasites on adult longevity in the polymorphic damselfly Mnais costalis Selys. *Ecological Entomology*, **29**, 361–366.

Tsubaki, Y., Hooper, R.E. & Siva-Jothy, M.T. (1997) Differences in adult and reproductive lifespan in the two male forms of *Mnais pruinosa costalis* Selys (Odonata : Calopterygidae). *Researches on Population Ecology*, **39**, 149–155.

Turnbull, C., Hoggard, S., Gillings, M., Palmer, C., Stow, A., Beattie, D. *et al.* (2011) Antimicrobial strength increases with group size: implications for social evolution. *Biology Letters*, **7**, 249–252.

Vainio, L., Hakkarainen, H., Rantala, M.J. & Sorvari, J. (2004) Individual variation in immune function in the ant *Formica exsecta*; effects of the nest, body size and sex. *Evolutionary Ecology*, **18**, 75–84.

Verhulst, S., Riedstra, B. & Wiersma, P. (2005) Brood size and immunity costs in zebra finches Taeniopygia guttata. *Journal of Avian Biology*, **36**, 22–30.

Visse, M., Sild, E., Kesler, M., Saks, L. & Hõrak, P. (2015) Do Atlantic salmon parr trade growth against immunity? *Marine and Freshwater Behaviour and Physiology*, **48**, 225–240.

Wilson, K. & Cotter, S.C. (2008) Density-dependent prophylaxis in insects. *Phenotypic Plasticity of Insects: Mechanisms and Consequences* (ed. by T. N. Ananthakrishnan and T. W. Whitman), pp. 381–420. Science Publishers Inc, Enfield, New Hampshire.

Wilson, K. & Reeson, A.F. (1998) Density-dependent prophylaxis: evidence from Lepidoptera–baculovirus interactions? *Ecological Entomology*, **23**, 100–101.

Wilson, K., Cotter, S.C., Reeson, A.F. & Pell, J.K. (2001) Melanism and disease resistance in insects. *Ecology Letters*, **4**, 637–649.

Wilson, K., Thomas, M.B., Blanford, S., Doggett, M., Simpson, S.J. & Moore, S.L. (2002) Coping with crowds: density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 5471–5475.

Wilson, K., Knell, R., Boots, M. & Koch-Osborne, J. (2003) Group living and investment in immune defence: an interspecific analysis. *Journal of Animal Ecology*, **72**, 133–143.

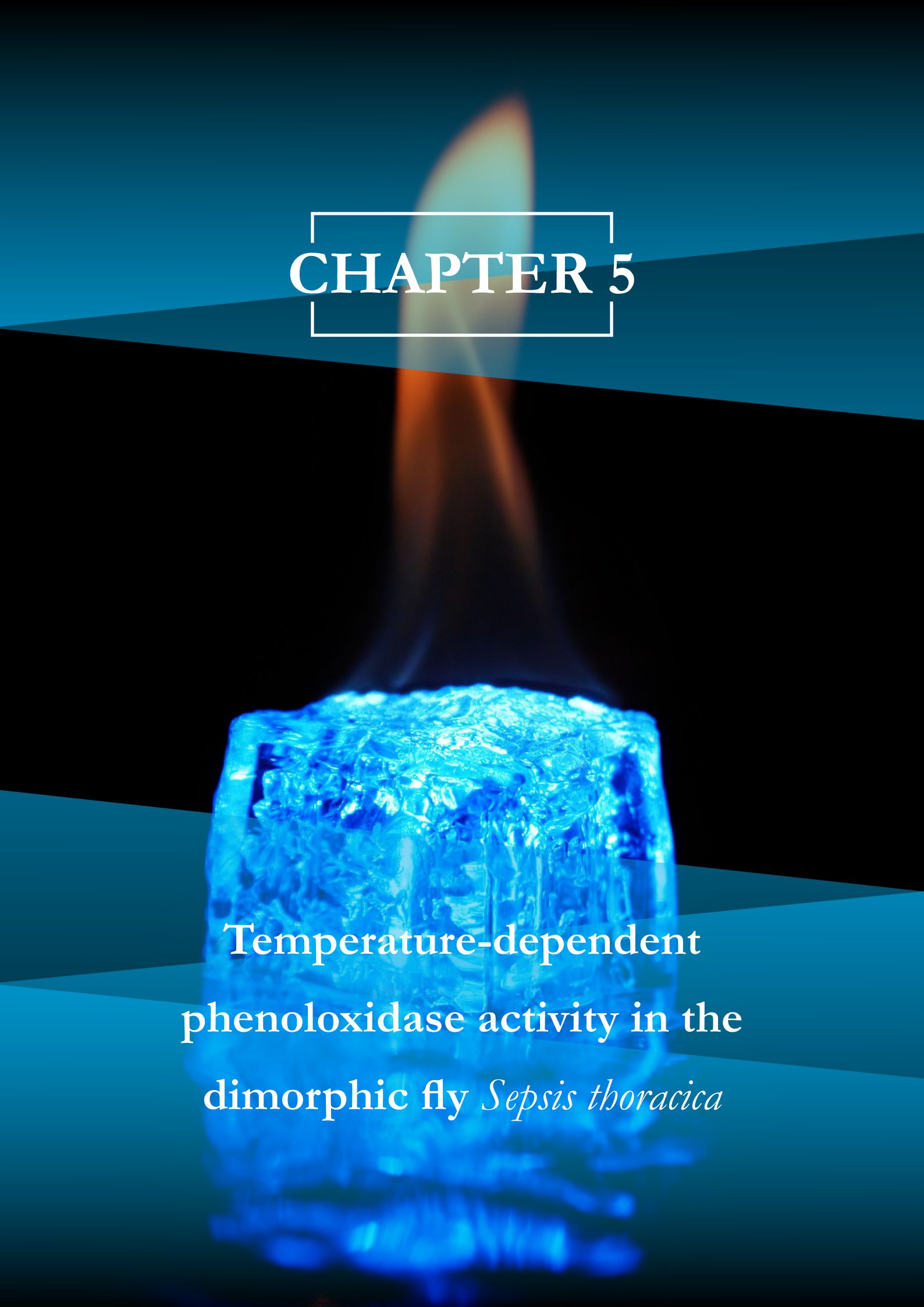
Yan, G., Severson, D.W. & Christensen, B.M. (1997) Costs and benefits of mosquito refractoriness to malaria parasites: implications for genetic variability of mosquitoes and genetic control of malaria. *Evolution*, **51**, 441–450.

Zera, A.J. & Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics*, **32**, 95–126.

Zuk, M. & Stoehr, A.M. (2002) Immune defense and host life history. *The American Naturalist*, **160**, S9–S22.

Accepted 16 March 2017

Associate Editor: Sheena Cotter



CHAPTER 5

Temperature-dependent phenoloxidase activity in the dimorphic fly *Sepsis thoracica*

CHAPTER 5

Manuscript Status: To be Submitted
Status in Journal: To be Submitted

Temperature-dependent phenoloxidase activity in the dimorphic fly *Sepsis thoracica*

Natalia Gourgoulianni [a], Wolf U. Blanckenhorn [a], Martin A. Schäfer [a], Juan Pablo Busso [a]

[a] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057, Zürich

ABSTRACT

Climate change alters both average temperatures as well as the frequency and severity of heat waves. Whereas numerous studies have investigated the effects of temperature on morphological and life history traits, current knowledge about temperature effects on immune function is still limited. We experimentally studied in the dimorphic dung fly Sepsis thoracica (Diptera: Sepsidae) how developmental temperature and larval density influences phenoloxidase (PO) activity, a key enzyme in insect pigmentation, thermoregulation and immunity, at the later adult stage. We raised flies from five latitudinal populations at three developmental temperatures (18, 24, 30). Developmental temperature strongly influenced PO activity, showing lowest values at 18°C, and altering the trade-off between body size and immunity. PO activity was further positively related to larval rearing density, possibly because of higher risks of pathogen infection or greater developmental stress due to stronger resource competition. Populations somewhat varied in PO activity, body size and coloration with no clear latitudinal pattern, so our results can be considered general and largely independent of the local climate the flies evolved in. Overall our results indicate that morph- and sex-specific PO activity, and likely immune function, in S. thoracica depends on temperature and larval density, modifying the existing trade-off relationship between immunity and body size. The strong dampening of the immune system of all morphs at cool temperatures suggests low-temperature stress in this warm-adapted species. Lastly, our results support the population density dependent prophylaxis hypothesis, which predicts higher investment in immunity due to limited resource availability and increased pathogen infection probability.

KEYWORDS

body size, density-dependent prophylaxis, Diptera, immunity, trade-off.

INTRODUCTION

Temperature is one of the most important ecological factors determining species diversity and abundance, and tolerance of thermal fluctuations is of prime importance for an individual's fitness (Kristensen et al., 2008). Since global warming poses a substantial challenge to many organisms (Parry et al., 2007; Sala et al., 2000; Thomas et al., 2004), the impact of temperature on co-adapted traits needs to be evaluated thoroughly. For example, an increase in temperature causes the decrease in body size in many ectotherms, known as temperature-size rule (Atkinson, 1994). The generality of the temperature size rule results from temperature differentially altering growth and developmental rates (Forster and Hirst, 2012; Forster et al., 2011; Zuo et al., 2012), which in turn is largely mediated by changes in metabolic rates (Hance et al., 2006; Van Baaren et al., 2010). Thus enzymatic activity and even protein structure can be significantly modified during thermal acclimation (Somero, 2004).

Temperature effects on enzymatic activity are not limited to metabolism, but also extend to the enzymes involved in the insect's immunity (Chown and Nicolson, 2004). Although in general a temperature increase should augment the rate of enzymatic reactions (Angilletta et al., 2010), effects on the immune system

are not straightforward, making it hard to predict how insect immunity will be affected by heat waves (Chaui–Berlinck et al., 2004). Whereas a number of studies document improved immune responses with increasing temperature (Adamo and Lovett, 2011), others find declines in some immune functions, e.g. melanization (Suwanchaichinda and Paskewitz, 1998).

In ectotherms, pigmentation is one key element linking thermal regulation, energy exchange, and immune responses. Darker pigmentation facilitates absorption of sunlight and helps increase the insect's body temperature (Lindstedt et al., 2009; Wasserthal, 1975; Watt, 1969). Thus darker individuals often attain higher fitness at cooler conditions if only by being more active at suboptimal temperatures (Clusella Trullas et al., 2007; Ellers and Boggs, 2002). Pigmentation differentiation among populations of different latitudes can be genetic (David et al., 1985). Although insect pigmentation is regulated by several genes, the phenoloxidase (PO) cascade is the most important enzymatic path leading to the formation of melanin (Söderhäll and Cerenius, 1998). PO catalyzes the oxygenation of monophenols to o-diphenols, and further oxidation of o-diphenols to o-quinones (Cerenius and Söderhäll, 2004). In addition to thermal regulation, melanin directly affects the innate immune response by acting as a physico-chemical barrier in the cuticle, thus blocking the invasion of pathogens (Armitage and Siva-Jothy, 2005; Barnes and Siva-Jothy, 2000; Cotter et al., 2004a). Melanin also adheres to pathogens, immobilizing and rendering them susceptible to other host defence effector mechanisms, including phagocytosis and encapsulation (Cerenius and Söderhäll, 2004; Siva-Jothy et al., 2005). Hence, pigmentation serves a double purpose in insects: it helps regulate the body temperature, and also has an important immune role.

Apart from temperature, the relative investment in immunity can be affected by additional factors such as resource availability, which strongly depends on population density (Agnew et al., 2002). If the fitness costs and benefits of possessing a trait vary according to conspecific density, then that trait should be phenotypically plastic with respect to density, provided that the organism is able to assess this (Via et al., 1995). Many insects are able to make such assessments during the larval stages and adjust their phenotype accordingly (Carroll and Dingle, 1996). A wide range of traits show such `density-dependent phase polyphenism', including coloration, wing and sensilla morphology, developmental rate, hormone secretion, metabolism, pheromone production, reproductive potential and mating behaviour (Applebaum and Heifetz, 1999). A potentially important life-history determinant showing density-dependent variation is the risk of infection. For most organisms, conspecifics are the main source of disease (Freeland, 1983). Contact with increasing numbers of conspecifics raises the probability of infection (Steinhaus, 1958), and hence the likelihood of needing to mount an immune response. For example, it has been demonstrated that larvae of the noctuid moth *Spodoptera exempta* are more resistant to their baculovirus when reared at high population densities. The activity in the haemolymph of phenoloxidase (PO), and a prominent phase polyphenism in the level of cuticular melanization (black larvae being more resistant than green larvae), were positively correlated with resistance (Reeson et al., 1998).

An excellent system to investigate the effects of temperature on phenotypic responses in life-history traits and immune function are black scavenger flies (Diptera: Sepsidae). Previous studies provided strong evidence that Sepsid flies respond both phenotypically and genetically to changes in mean and fluctuating developmental temperature regimes (Berger et al., 2013; Blanckenhorn, 1997). These flies have short development times and follow the temperature-size rule (Berger et al., 2013). We focused on *Sepsis thoracica* because of its wide geographic distribution (Europe, Africa, Australasia), permitting comparisons of different populations that presumably evolved under a broad range of thermal regimes. Most importantly, while most sepsid species are rather uniform in pigmentation, males of *S. thoracica* exhibit two morphs: obsidians (small and black) and ambers (large and yellow), while the females are always black (Busso et al., 2017). The different morphs also vary in immunological properties, with obsidians showing greater PO activity than ambers (Busso et al., 2017). In this study, we raised flies from five latitudinal populations at three developmental temperatures in the laboratory to measure phenotypic responses in body size, coloration and PO activity. In addition, we used flies that developed at different rearing densities to investigate how this will affect the PO activity and hence the immune efficiency (May, 1979; Renault et al., 2003; Scharf et al., 2014).

MATERIALS AND METHODS

Origin of flies and culture

S. thoracica used for our common garden experiment originated from five populations sampled across Europe: Ludwigshafen, Germany (49.48° N, 8.42° W), Borgonuovo, Italy (46.33° N, 9.44° W), Asturias, Spain (43.3° N, 6.0° E), Petroia, Italy (43.23° N, 12.56° W), and Padula, Italy (40.34° N, 15.66°

W). Laboratory populations were maintained as iso-females to avoid loss of allelic variation within populations or adaptation to laboratory conditions. Fly cultures were maintained in climate chambers at 18 ± 1 °C under a 14:10 h light:dark cycle. Iso-female lines were maintained in plastic containers and provided with sugar, water and cow dung for oviposition material. Containers and contents were renewed once a month.

Experimental design

To obtain sufficient flies from each iso-female line, three plastic dishes (43 x 15 x 15 mm) with previously frozen fresh cow dung were placed for 24h in the containers of four to five iso-female lines per population to collect sufficient eggs. All lids were then placed into glass vials (10 x 4 x 4 cm) containing sugar to ensure that emerging flies had immediate access to food resources. Dishes were then randomly assigned to one of three developmental temperatures (18, 24, 30 ºC), such that all lines of each population was represented at all temperatures. Due to the difficulty of directly manipulating and assessing larval density in the dung without damaging the larvae or affecting their immune system, we used the number of emerged adults as a proxy of larval competition. Random subsets of males and females were collected four days after emergence and stored at -80 ºC until used for our morphometric and immunological measurements. (The same flies were used for both.) In total, we scored 876 individuals of both sexes and both male colour morphs.

Emerged adult individuals were dissected and their foreleg femur was photographed under a stereo microscope (MZ12 with a Leica DFC490 camera) against a neutral white background. The camera was calibrated with a mini IT-8 calibration target to guarantee colour consistency across pictures. The target also served as a scale to posteriorly measure size. We used a self-written code in ImageJ, which measured the amount of pixels of the foreleg femur, and converted them to the real area according to the scale correspondent to each picture. The code also measured the amount of melanic pixels in the femur. Any pixel with a V value over 163 in the YUV colourspace was defined as melanic. This threshold was selected based on the valley of the bimodal distribution of the V values present in the femur colouration. Melanism represented the proportion of black and brown pixels over all the pixels of the femur.

Immunological assessment

To assess PO activity and total protein content, individuals were homogenized with 80 µL of cold phosphate buffered saline (PBS) in the TyssueLyser. Samples were then expelled to a chilled centrifuge (5 min, 5°C, 13000 rpm) in a spin column (M1003, Mobicol) with a 10 µm pore filter (M2210, Mobicol). Total PO activity was measured based on 20µl of each sample added to 20ml PBS. We then added 7µl of bovine pancreas α-chymotrypsin (Sigma C7762, 2 mg/mL of ddH2O), whereafter the plate was incubated for 10 min at room temperature (α-chymotrypsin catalyzes the conversion of inactive PO into its active form, allowing all body fluid-bound PO to be used in the analysis). After incubation, 133 µl of water and 10 µl L- DOPA (Sigma-Aldrich) was added to each sample within a flat-bottom 96-well plate. PO activity was quantified using a microplate reader (SpectraMax 340PC384, Molecular Devices) and defined as the total change in absorbance over six hours at 490nm ($A490$). Samples were randomly placed on the plate with regard to temperature, treatment, sex/morph and rearing density. As the PO reaction proceeds, absorbance increases because of the formation of melanin. Individuals with a greater amount of total PO in their body fluid were expected to exhibit a greater change in absorbance ($\Delta A490$). As physiological control we applied the same procedure, but using only cold PBS without fly extract (see similar procedures in Cotter et al., 2004b; González-Santoyo and Córdoba-Aguilar, 2012).

To estimate total protein concentration, 5µl from each sample diluted in 45µl PBS and added to a separate well of a flat-bottom 96-well plate. For the protein concentration measurement, we used albumin standards (from 2000 to 50 mg/mL) containing 150µl of a bicinchoninic acid (BCA) mix (BCA Protein Assay Kit 23225, Thermo Scientific). The total protein concentration of the samples was compared to a standard with known protein concentration. Protein concentration was measured with a microplate reader at 590nm for 9 minutes.

Statistical analyses

We calculated the slope of the maximum velocity (Vmax) of the reaction during the linear phase (inflection point) for each individual using a five-parameter logistic regression model (Gottschalk & Dunn 2005). In order to control for the autonomous conversion of L-Dopa to Dopaquinone, we subtracted the PO activity measured in the control essays. For each fly we then calculated the Vmax per total protein amount to standardize for protein concentration of the solutions. These values (Vmax/µg protein) were used for subsequent statistical analysis.

To analyse the effects of temperature and larval

density on body size we fitted a generalized mixed effects model. In the model we included morph (3 levels: amber, obsidian and female). Morph and temperature were used as fixed factors and population density as a covariate. We ran a full factorial analysis including all two-way and the three-way interactions. Iso-female lines nested within populations were incorporated as random effect.

To analyse the influence of the various variables on PO activity we also employed generalized mixed effects models. The model included the predictors morph, body size, larval density, temperature and population as fixed factors. Again, iso-female line was incorporated as random effect. We further included all two-way and three-way interactions among the predictor variables. Non-significant interactions (P>0.1) were removed from the final model, which did not affect the statistical significance of the main effects and ensuring that our results are robust. All analyses were performed with the software R Version 3.2.2 (R Core R Development Core Team 2015).

RESULTS

As previously known and expected, body size differed between the two male morphs (Table 1), with obsidians being significantly smaller than ambers. Female body size approaches the obsidians' size (Fig. 1). Body size decreased with increasing temperature, although the extent differed between morphs as indicated by a significant two-way interaction (Table 1), with ambers decreasing the most relative to obsidians and females (Fig. 1). A similar, marginally non-significant (p= 0.053; Table 1) interaction was

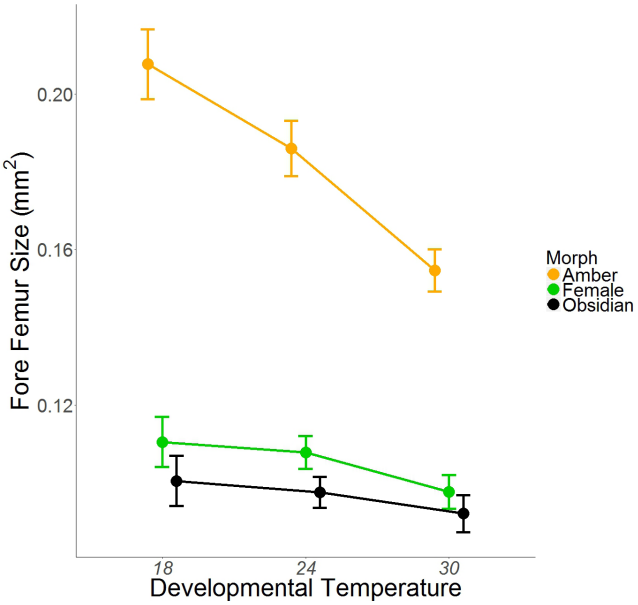


Figure 1. Mean ± 95% CI effect of temperature on body size of both sexes and male morphs.

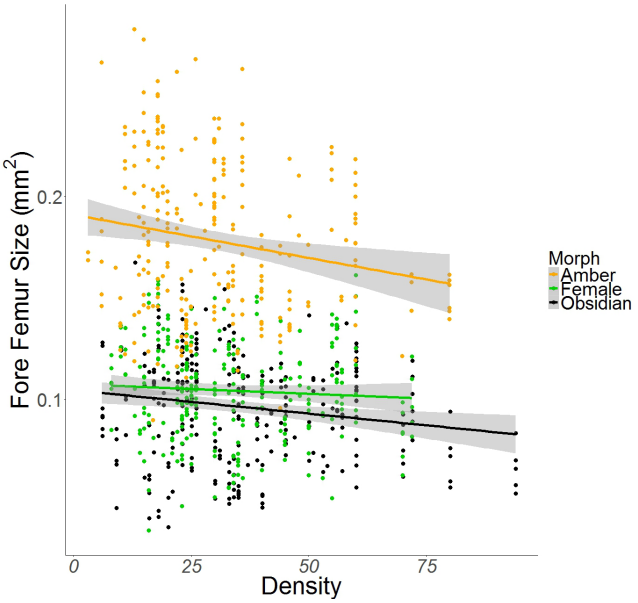


Figure 2. Effect of larval density on body size of both sexes and two male colour morphs.

present for morph and density, as ambers tended to reduce body size more strongly with density than females and obsidians (Fig. 2).

With a mixed effects model we tested for the effects of morph, temperature, density, population and body size on PO activity. Morphs differ in PO activity, but this effect depends on developmental temperature and density (Table 2, Fig. 3). The model indicates that while the morphs and females do not differ at 18 °C, they do so at 24°C and 30°C, in agreement with previous findings (Busso et al., 2017). At the latter two higher temperatures obsidians have the highest PO activity, followed by females and, lastly, ambers. The PO activity for all groups at these latter two temperatures is also higher than at 18°C.

Except at 18°C, the effect of body size on PO activity varies between morphs and also depends on the ambient temperature (Table 2, Fig. 4). While 18°C presents close to zero slopes for both morphs and females, at 24°C and 30°C we observe a decrease in PO activity with body size in both obsidians and females, but not in the ambers. In addition, we found that PO activity is positively affected by rearing density, and the magnitude of this effect differs between populations (Table 2, Fig. 5).

Finally, we obtained some additional population differences in PO activity, which however were weak in comparison and do not follow an expected latitudinal trend (Table 2, Fig. S1).

DISCUSSION

In the dimorphic black scavenger fly *S. thoracica*, we investigated the effects of developmental

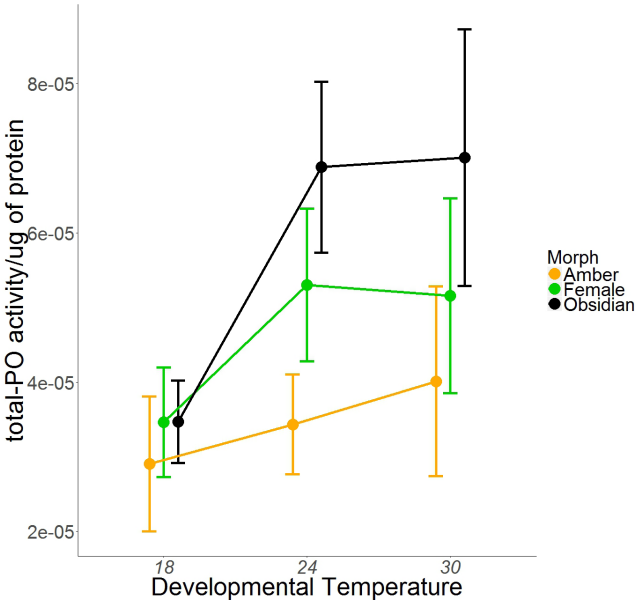


Figure 3. Mean ± 95% CI total PO activity for each morph at the three developmental temperatures.

temperature, rearing density and geographic origin on the phenoloxidase (PO) activity of alternative male morphs and females. The results provided three main conclusions. First, temperature strongly influences PO expression, being lowest at 18°C for all individuals of all populations. Secondly, the trade-off between body size and immune investment is actually conditional upon the developmental temperature, being absent at 18°C, where PO activity was at its lowest, but present at higher temperatures. Thirdly, PO activity is affected by rearing density, and this influence depends somewhat on the population of origin, however not following a latitudinal trend.

In ectotherms, cellular and enzymatic processes are particularly temperature-sensitive due to the higher plasticity of the three-dimensional configuration of their proteins (Somero, 2004). Hence, it was expected that insect immunity would decline with temperature (Fig. 3). The strong dampening effect of cool temperature (18°C) on the immunity of the flies, especially for the all-black females and obsidian males, very likely results from cold being a general abiotic stressor for insects (Clark and Worland, 2008). It could further result because of increased energy demands necessary for improving cold tolerance (Sinclair, 2015), which conflicts with immune activity (Linderman et al., 2012). The sharp decrease in immunity at 18°C concurs with what has been observed in *Gryllus veletis*, where PO activity also declined drastically at cooler temperature (Ferguson et al., 2016). Pathogen growth normally increases when warmer (Harvell et al., 2002), hence it is logical to expect higher PO activity at higher temperatures.

Consistent with previous findings (Busso et al.,

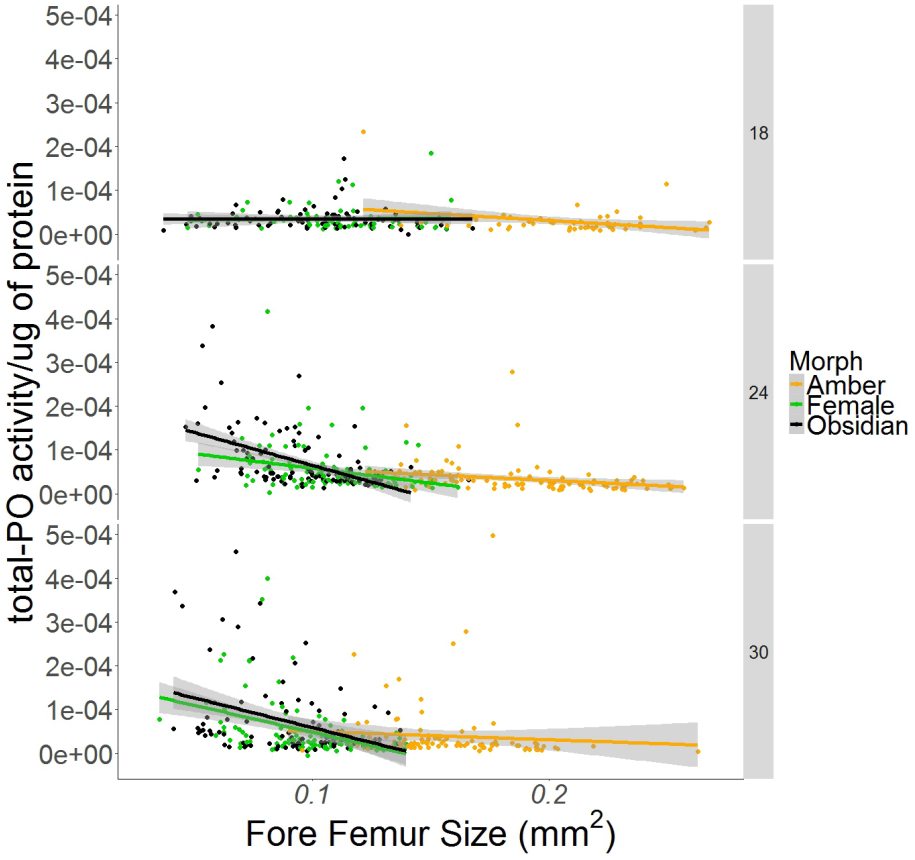


Figure 4. Effects of body size and temperature on PO activity in females and both male morphs.

Table 1. Wald-Tests for the effects of population, temperature, density, morph on body size in *Sepsis thoracica.* Significant P-values are given in bold.

Body size	df	Den df	F-value	P-value
Morph	2	1169.52	222.14	**<0.001**
Temperature	2	1164.63	5.89	**0.002**
Density	1	1118.56	46.574	**<0.001**
Temperature x Morph	4	1167.6	3.139	**0.013**
Density x Morph	2	1169.29	3.261	0.053
Temperature x Density	2	1157.3	0.144	0.865
Temperature x Density x Morph	4	1168.76	1.551	0.185

2017) our results show that the two colour morphs differ in PO-activity with obsidians showing higher enzymatic activity than ambers, except at 18°C, where there was no difference between morphs. Moreover, we further confirmed the trade-off between immunity and body size in obsidians and females, but not in ambers (Fig. 4). However, this trade-off was observed only at 24°C and 30°C, while the low PO activity at 18°C did not show a negative relationship signifying any trade-off. It is likely that at 18°C traits related to cold resistance gain priority relative to immunity, such that the trade-off between immunity and body size is actually conditional upon developmental temperature. Alternatively, flies reach their largest size (within our setting) at 18°C, according to the temperature-size-rule (Atkinson, 1994); hence, it could be that the investment in body size at this temperature contributes to compromising the investment in immunity. Either factor or a both combined would result in a decrease in immunity.

In addition to the immunity/body size trade-off, we also found that the larval rearing density can affect PO activity as well, though this effect varied quantitatively (but not qualitatively) between populations. According to the density 'dependent prophylaxis' hypothesis (Wilson and Reeson, 1998), individuals reared at high density should invest more in the expression of immunogenes due to greater expected competition and higher infection propability at the adult stage. Alternatively, high rearing density could lead to a weaker investment in immune function as a consequence of the more stressful environment during early life. Our results support the first hypothesis, indicating that high larval densities lead to higher investment in the immune system in three out of five poplations and hence overall (Fig. 5). Since the breeding substrate in *S. thoracica* is dung, on which

Table 2. Wald Chi^2-Tests for the effects of population, temperature, density, morph and body size on total phenoloxidase activity in *Sepsis thoracica.* Significant P-values are given in bold.

Total PO activity	df	error df	F-value	P-value
Morph	2	55	3.827	**0.028**
Temperature	2	7.9	13.340	**0.003**
Density	1	61.3	13.360	**<0.001**
Body size	1	58.3	61.950	**<0.001**
Morph x Temperature	4	45.8	1.268	0.297
Morph x Density	2	63	1.187	0.315
Morph x Body Size	2	724	1.932	0.146
Area x Temperature	2	50.8	7.081	**0.002**
Temperature x Density	2	28.6	1.961	0.159
Body size x Density	1	41.2	0.472	0.496
Morph x Temperature x Density	4	485.1	1.963	0.099
Morph x Temperature x Body size	4	667.3	2.534	**0.039**
Population	4	6.2	4.197	0.056
Population x Morph	8	43	1.881	0.089
Population x Temperature	8	6.4	4.370	**0.040**
Population x Density	4	27.8	2.739	**0.049**
Population x Body size	4	53.1	0.898	0.472

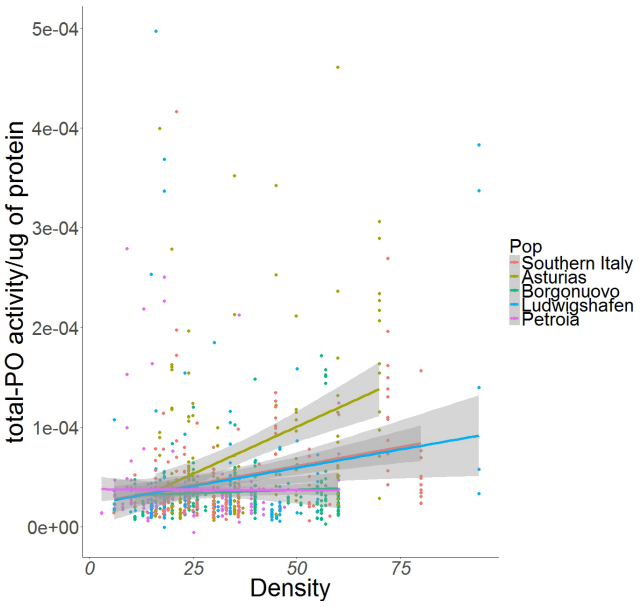


Figure 5. Population differences in PO activity in *S. thoracica*.

fungi, parasitic mites and various pathogens aggregate, the propabilities of infection are expected to be high, especially upon adult emergence from the pupa, when the flies are soft and vulnerable. Our results concur with what has been found in various social and non-social arthropods including orthopterans, lepidopterans, thysanopterans, coleopterans and hymenopterans, among others (Barnes & Siva-Jothy 2000; Wilson *et al.* 2002; Cotter *et al.* 2004; Stow *et al.* 2007; Turnbull *et al.* 2011)as population density increases, so too does the per capita risk of becoming infected. Under such circumstances, natural selection should favor individuals that use cues associated with population density to determine the optimal allocation of resources to disease resistance mechanisms. As a consequence, individuals experiencing crowded conditions are predicted to be more resistant to parasites and pathogens than those experiencing low-density conditions. This phenomenon (termed \"density-dependent prophylaxis\" but see (Wilson *et al.* 2003; González-Tokman *et al.* 2014)group living is normally thought to lead to an increased exposure to parasitism. As a consequence, it is predicted that animals living in groups will invest more resources (energy, time, risk, etc.. Consequently, it is conceivable that in *S. thoracica* increased competition at high larval densities challenge individuals to invest more rescources into immunity than growth and development, producing density-dependent prophylaxis at later stages in life.

The interpopulation variation observed at different temperatures and densities does not follow any latitudinal gradient, contrary to expctation. Hence, evolution of the thermal optimum of the immune response does not seem to relate strongly with the thermal profile in which the flies evolved. The small number of populations sampled in our study captures variation between them, but does not allow us to address the actual factors underlying those differences. It is possible that several other environmental factors that strongly affect the melanism / body size trade-off (see Chapter 1) could influece the insects' immunity as well. Future studies assessing characteristics such as pathogen abundance and identity at each location might suggest possible factors mediating the interpopulation differences in immunity.

In conclusion, our experimental study of PO-activity in *S. thoracica* indicates that the trade-off relationship between immunity and body size is significantly affected by the developmental temperature. The consistently low PO activity at 18°C observed in all our populations suggests a species effect that is largely independent of the local climate the flies evolved in. In addition, our results support morph-dependent PO investment according to a size-immunity trade-off (Busso et al., 2017), as well as the density dependent prophylaxis hypothesis, which predicts higher investment in immunity due to limited resource availability and increased pathogen infection probability. Challenging the immune system in *S. thoracica* by artificially or naturally inducing pathogen infections and relating activated PO-expression to fitness would be a logical next step to further understand the adaptive significance of the great phenotypic plasticity in body size and coloration in this species, but also how immunoresponses evolve in response to climate change.

REFERENCES

Adamo SA, Lovett MM, 2011. Some like it hot: the effects of climate change on reproduction, immune function and disease resistance in the cricket Gryllus texensis. Journal of Experimental Biology 214:1997-2004.

Agnew P, Hide M, Sidobre C, Michalakis Y, 2002. A minimalist approach to the effects of density-dependent competition on insect life-history traits. Ecological Entomology 27:396-402.

Angilletta MJ, Cooper BS, Schuler MS, Boyles JG, 2010. The evolution of thermal physiology in endotherms. Front Biosci E 2:861-881.

Applebaum S, Heifetz Y, 1999. Density-dependent physiological phase in insects. Annual review of entomology 44:317-341.

Armitage SAO, Siva-Jothy MT, 2005. Immune function responds to selection for cuticular colour in *Tenebrio molitor*. Heredity 94:650-656.

Atkinson D, 1994. Temperature and Organism Size: A Biological Law for Ectotherms?: Academic Press.

Barnes AI, Siva-Jothy MT, 2000. Density–dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. Proceedings of the Royal Society of London Series B: Biological Sciences 267:177-182.

Berger D, Postma E, Blanckenhorn WU, Walters RJ, 2013. Quantitative genetic divergence and standing genetic (co)variance in thermal reaction norms along latitude. Evolution 67:2385-2399.

Blanckenhorn WU, 1997. Altitudinal life history variation in the dung flies Scathophaga stercoraria and *Sepsis cynipsea*. Oecologia 109:342-352.

Carroll SP, Dingle H, 1996. The biology of post-invasion events. Biological Conservation 78:207-214.

Cerenius L, Söderhäll K, 2004. The prophenoloxidase-activating system in invertebrates. Immunological reviews 198:116-126.

Chaui–Berlinck JG, Navas CA, Monteiro LHA, Bicudo JEPW, 2004. Temperature effects on a whole metabolic reaction cannot be inferred from its components. Proceedings of the Royal Society of London B: Biological Sciences 271:1415-1419.

Chown SL, Nicolson S, 2004. Insect physiological ecology: mechanisms and patterns: Oxford University Press.

Clark MS, Worland MR, 2008. How insects survive the cold: molecular mechanisms—a review. Journal of Comparative Physiology B 178:917-933.

Clusella Trullas S, van Wyk JH, Spotila JR, 2007. Thermal melanism in ectotherms. Journal of Thermal Biology 32:235-245.

Cotter SC, Hails RS, Cory JS, WIlson K, 2004a. Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. Journal of Animal Ecology 73:283-293.

Cotter SC, Kruuk LEB, Wilson K, 2004b. Costs of resistance: genetic correlations and potential trade-offs in an insect immune System. Journal of evolutionary biology 17:421-429.

David J, Capy P, Payant V, Tsakas S, 1985. Thoracic trident pigmentation in *Drosophila melanogaster*: differentiation of geographical populations. Genet Sel Evol 17:211-224.

Ellers J, Boggs CL, 2002. The evolution of wing color in *Colias* butterflies: heritability, sex linkage, and population divergence. Evolution 56:836-840.

Ferguson LV, Heinrichs DE, Sinclair BJ, 2016. Paradoxical acclimation responses in the thermal performance of insect immunity. Oecologia 181:77-85.

Forster J, Hirst AG, 2012. The temperature-size rule emerges from ontogenetic differences between growth and development rates. Functional Ecology 26:483-492.

Forster J, Hirst AG, Atkinson D, 2011. How do organisms change size with changing temperature? The importance of reproductive method and ontogenetic timing. Functional Ecology 25:1024-1031.

Freeland W, 1983. Parasites and the coexistence of animal host species. The American Naturalist 121:223-236.

González-Santoyo I, Córdoba-Aguilar A, 2012. Phenoloxidase: a key component of the insect immune system. Entomologia Experimentalis et Applicata 142:1-16.

Hance T, Van Baaren J, Vernon P, Boivin G, 2006. Impact of extreme temperatures on parasitoids in a climate change perspective. Annual review of entomology 52:107.

Harvell CD, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD, 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296:2158-2162.

Kristensen TN, Hoffmann AA, Overgaard J, Sørensen JG, Hallas R, Loeschcke V, 2008. Costs and benefits of cold acclimation in field-released *Drosophila*. Proceedings of the National Academy of Sciences 105:216-221.

Linderman JA, Chambers MC, Gupta AS, Schneider DS, 2012. Infection-Related Declines in Chill Coma Recovery and Negative Geotaxis in Drosophila melanogaster. PLOS ONE 7:e41907.

Lindstedt C, Lindström L, Mappes J, 2009. Thermoregulation constrains effective warning signal expression. Evolution 63:469-478.

May ML, 1979. Insect Thermoregulation. Annual Review of Entomology 24:313-349.

Parry M, Canziani OF, Palutikof JP, van der Linden PJ, Hanson CE, 2007. Climate change 2007: impacts, adaptation and vulnerability: Cambridge University Press Cambridge.

Reeson AF, Wilson K, Gunn A, Hails RS, Goulson D, 1998. Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. Proceedings of the Royal Society of London B: Biological Sciences 265:1787-1791.

Renault D, Hance T, Vannier G, Vernon P, 2003. Is body size an influential parameter in determining the duration of survival at low temperatures in *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae)? Journal of Zoology 259:381-388.

Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, 2000. Global biodiversity scenarios for the year 2100. science 287:1770-1774.

Scharf I, Sbilordo SH, Martin OY, 2014. Cold tolerance in flour beetle species differing in body size and selection temperature. Physiological Entomology 39:80-87.

Sinclair BJ, 2015. Linking energetics and overwintering in temperate insects. Journal of Thermal Biology 54:5-11.

Siva-Jothy MT, Moret Y, Rolff J, 2005. Insect immunity: an evolutionary ecology perspective. Advances in insect physiology 32:1-48.

Söderhäll K, Cerenius L, 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Current opinion in immunology 10:23-28.

Somero GN, 2004. Adaptation of enzymes to temperature: searching for basic "strategies". Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139:321-333.

Steinhaus EA, 1958. Crowding as a possible stress factor in insect disease. Ecology 39:503-514.

Suwanchaichinda C, Paskewitz SM, 1998. Effects of larval nutrition, adult body size, and adult temperature on the ability of *Anopheles gambiae* (Diptera: Culicidae) to melanize Sephadex beads. Journal of Medical Entomology 35:157-161.

Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, Collingham YC, Erasmus BF, De Siqueira MF, Grainger A, Hannah L, 2004. Extinction risk from climate change. Nature 427:145-148.

Van Baaren J, Le Lann C, Van Alphen JJ, 2010. Consequences of climate change for aphid-based multi-trophic systems. Aphid Biodiversity under Environmental Change: Springer. p. 55-68.

Via S, Gomulkiewicz R, De Jong G, Scheiner SM, Schlichting CD, Van Tienderen PH, 1995. Adaptive phenotypic plasticity: consensus and controversy. Trends in Ecology & Evolution 10:212-217.

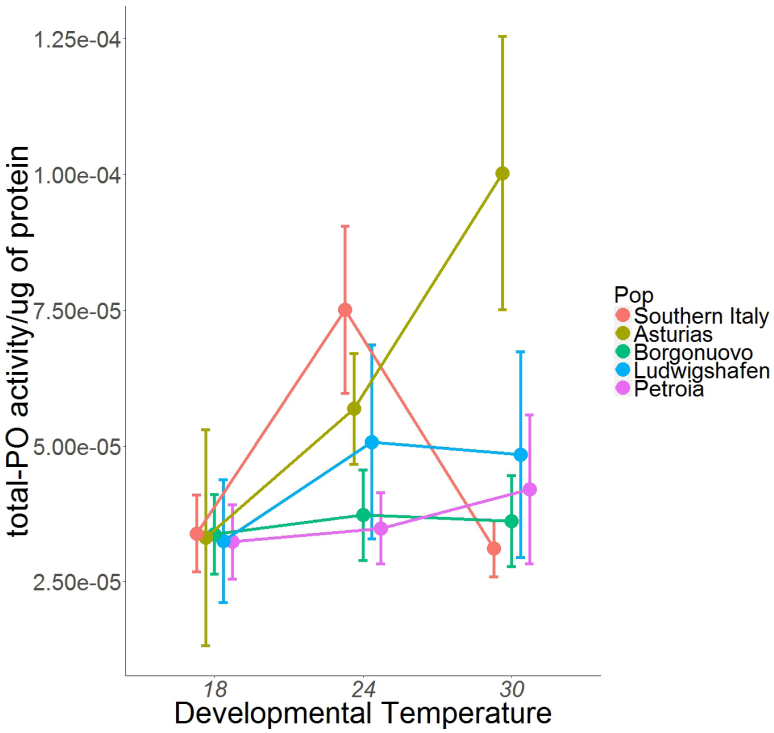
Wasserthal LT, 1975. The rôle of butterfly wings in regulation of body temperature. Journal of Insect Physiology 21:1921-1930.

Watt WB, 1969. Adaptive significance of pigment polymorphisms in *Colias* butterflies, II. Thermoregulation and photoperiodically controlled melanin variation in *Colias eurytheme*. Proceedings of the National Academy of Sciences 63:767-774.

Wilson K, Reeson AF, 1998. Density-dependent prophylaxis: evidence from Lepidoptera–baculovirus interactions? Ecological Entomology 23:100-101.

Zuo W, Moses ME, West GB, Hou C, Brown JH, 2012. A general model for effects of temperature on ectotherm ontogenetic growth and development. Proceedings of the Royal Society B: Biological Sciences 279:1840-1846.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Mean ± 95% CI total PO activity for each population at the three temperatures.

CONCLUSIONS & FUTURE DIRECTIONS

CONCLUSIONS

This dissertation analyzed the trade-off between body size and melanism from multiple perspectives, revealing the possible role of several environmental factors affecting various fitness components important in the origin and maintenance of the trade-off. These studies shed light on intraspecific processes that can dramatically shape the phenotype of a species and could conceivably constitute the beginning of a speciation process.

One of the foremost striking results of this study is the discovery of the polymorphism, or polyphenism (because it is largely environmentally induced), of *S. thoracica* males resulting from a threshold trade-off between body size and melanism, a phenomenon that is relatively rare in nature but has been reported in some other species and contexts (Hodjat, 1970; Lee and Wilson, 2006). The sigmoid relationship between these two traits divides the males' phenotypic landscape into two nearly discrete morphs: obsidian (small and black) and amber (large and orange). This dimorphism was found in all iso-female lines of all the 15 European populations we sampled, from southern Italy to Estonia. In **Chapter 1**, we found that latitude influences melanism but not body size in *S. thoracica* males, while other climatic variables further contribute to the geographic differentiation among populations that can be interpreted as adaptive. The sigmoid trade-off between body size and melanism in *S. thoracica* males not only defines the plasticity of male phenotypes, but is also plastic itself. The trade-off itself seems to evolve across populations in response to various climatic factors such as temperature, insolation and UV radiation, suggesting that plasticity has an underlying genetic basis. As the plastic and evolutionary responses to temperature (and other factors) differed from one another, plasticity does not necessarily follow the direction of evolution of the trade-off, but rather adds to it. To our knowledge, this is the first study addressing the influence of climatic factors on plastic trade-offs, evincing their possible role in the evolution of polymorphisms. However, environmental factors are not playing an isolated role in this species. These effects are accompanied by the influence of biological components that also impact the morphospace of the species.

Chapter 2 documents that intraspecific disruptive sexual selection on male body size is mediated by male-male interactions, also seen in dung beetles (Moczek and Emlen, 1999; Moczek and Emlen, 2000). This disruptive selection likely contributes to the origin and/or maintenance of the discrete male morphs in this species, together with the other processes described in the framework of this thesis. Due to the elusive ghost of evolution past, we of course can never know for certain whether the current selection pressures are indeed identical to those that mediated the origin of the described polymorphism. We additionally found significant positive linear sexual selection on body size that interacted with selection on coloration, probably also playing an important role in the origin and/or maintenance of the threshold relationship between the two traits. The simultaneous existence of disruptive selection and polymorphism in *S. thoracica* supports the role of sexual selection in the intraspecific diversification of traits (Andersson, 1994; Eberhard, 2010), which could be a starting point for speciation (Corl et al., 2010; Wittkopp et al., 2009). Intraspecific disruptive selection is of major interest in this context because it theoretically strengthens reproductive isolation and sympatric speciation by sexual selection, a controversial phenomenon in evolutionary biology (Gavrilets and Hayashi, 2005; van Doorn et al., 2004).

However, the reproductive fitness landscape shows clearly that the male morphs do not occupy the highest peaks of the landscape. This small mismatch suggests that although sexual selection significantly shapes the trade-off, it does not act in isolation. Adding to the previous effects, in **Chapter 3** we found that (adult) body size is generally under positive selection by various invertebrate predators, whereas selection on male coloration varied significantly among predator species and with background color. Several studies have shown that arthropod predators can have strong effects on insect populations (Fagan and Hurd, 1994; Kristensen, 1994; Lang et al., 1999; Wooster, 1994); consequently, the positive directional selection exerted by arthropods likely is an important force driving the evolution of body size in invertebrates. However, given the myriad of predators of any given prey species, the corresponding net viability selection resulting in the wild can only be assessed comprehensively in a phenomenological way (by taking the prey perspective), but not in a mechanistic or functional manner as

done here. Although we found positive selection on body size and negative selection on coloration, these forces did not modify the sigmoid curve between body size and coloration.

In **Chapter 4**, we discovered that obsidians have higher total phenoloxidase (PO) activity than ambers. We found a negative relationship between total-PO activity and body size in females and obsidians but not ambers, suggesting that growth and immunity are more costly for the former. Hence, the body size melanism trade-off also translates into a body size immunity trade-off. These results were further expanded in **Chapter 5**, which foremost shows that the trade-off between body size and immunity not only depends on the morph (an intrinsic characteristic), but also on temperature (an extrinsic characteristic). The cool 18^{o}C treatment reduced the immunity of all individuals to a minimum regardless of morph, population density or body size. This drastic effect of temperature highlights that any trade-off can be modified by the environment. In contrast to **Chapter 4**, population density negatively affected PO activity in **Chapter 5**, as predicted by the density-dependent prophylaxis hypothesis, an effect that additionally depended on the population of origin. Hence, external factors not only influence the evolution of a trade-off, but also affect trait responses to environmental factors.

In conclusion, I found that a sigmoid trade-off between two traits can divide the male phenotypic landscape into two almost discrete morphs, producing an intraspecific polyphenism. This trade-off is the result of a plastic response to the environment, allowing all genotypes to accommodate its phenotype to the current conditions in which it develops, thus augmenting its fitness. This trade-off not only represents plasticity, but it is also plastic itself. The trade-off thus presents an underlying genetic (GxE) component which allows the sigmoid relationship between size and coloration to evolve across populations in response to the prevailing environment. Hence, this polyphenism seems to be the result of numerous interacting environmental and biological factors that in synergy affect the fitness of the individuals. While obsidians would have a greater advantage when facing immunological challenges, ambers would have higher survival against predators and a higher mating success. This dissertation provides good evidence that trade-offs between different traits can result in alternative resource allocation strategies, even within one species. These strategies can produce strikingly different alternative phenotypes, evincing that there is not only one optimal solution to address fitness optimization (Farnsworth and Niklas, 1995).

FUTURE DIRECTIONS

This dissertation presented comprehensive analyses evincing the complex network of biological and environmental elements influencing trade-offs and their evolution. Although it is an extensive study analyzing the matter from multiple angles, it is not fully inclusive and hence serves as an initial building block to further advance this research from several other perspectives.

Since both body size and melanism are resource demanding (Reichle, 1968; Talloen et al., 2004), it would be interesting to further analyze in more detail how resource (dung) limitation influences the relative investments into both traits. Food availability in insects strongly influences adult body size, which is primarily determined by the minimal viable weight and the critical weight (Mirth and Riddiford, 2007; Nijhout, 2003; Reichle, 1968). Food also drastically influences the immune response of insects (Lee and Wilson, 2006). Hence, possible experiments manipulating food amounts would uncover the decision-making process underlying resource allocation to each trait in the juvenile growth stage. Moreover, such work would permit estimation of the heritability of the traits involved in the trade-off as well as the relative importance of genetic vs. environmental factors (i.e. GxE, the genetic component of plasticity). As already demonstrated in **Chapters 1 & 5,** with a factorial design additionally including temperature such assessments could further determine the relative roles of different environmental components in the evolution of the two traits and the trade-off between them.

In **Chapter 2** we observed a strong role of male-male competition on sexual selection acting on this trade-off. The morphological and behavioral differences between the morphs indicate at least the beginning of alternative reproductive strategies in *S. thoracica*. Future studies should study mating behavior in the field to determine if the different morphs employ either the same or indeed alternative reproductive tactics (Taborsky and Brockmann, 2010). Estimating mating success of the different morphs in nature would address the overlap between sexual selection measured in the laboratory and the field, and hopefully validate the results presented here. Moreover, to identify further behavioral differences between the two morphs, it would be useful to analyze the predator evasion behaviors of each morph underlying the predation results reported in **Chapter 3**. Future studies employing object tracking software would allow quantification of potentially subtle and quantitative (rather than qualitative) behavioral differences between morphs contributing to differential survival.

However, as highlighted before predators do not

act in isolation on the survival component of fitness. Pathogens also play an important role in black scavenger flies due to their life-history traits and habits being intimately related to animal excrements, a pathogen-rich environment. Although **Chapters 4 & 5** documented different responses of the *S. thoracica* morphs involving phenoloxidase, it would be interesting to experimentally or naturally challenge their immune system to induce pathogen infections, and to relate phenoloxidase activity of the individuals to their survival and reproduction more directly (González-Santoyo and Córdoba-Aguilar, 2012; Iwanaga and Lee, 2005; Schmid-Hempel, 2005; Strand, 2008). This would further allow partitioning the relative effects of body size and immunity on overall longevity and pathogen resistance.

Finally, gathering all these various fitness components would allow the construction of an integrated fitness model. The analyses of the simultaneous influence of all factors in the fitness landscape of the male morphs should ultimately clarify the relative roles of the various factors on the maintenance of the two male morphs in nature. It would also allow the development of computational evolutionary models to help understand why the relationship between body size and melanism/immunity took a sigmoid shape, which is relatively rare, rather than any other possible (e.g. the more common linear) relationship. While my dissertation research covered a single, for many people possible obscure and uniteresting sepsid fly species, the researched phenomena are nevertheless more common and general in nature.

REFERENCES

Andersson B, 1994. Sexual Selection: Princeton University Press.

Corl A, Davis AR, Kuchta SR, Sinervo B, 2010. Selective loss of polymorphic mating types is associated with rapid phenotypic evolution during morphic speciation. Proceedings of the National Academy of Sciences of the United States of America 107:4254-4259.

Eberhard WG, 2010. Evolution of genitalia: theories, evidence, and new directions. Genetica 138:5-18.

Fagan WF, Hurd L, 1994. Hatch density variation of a generalist arthropod predator: population consequenes and community impact. Ecology:2022-2032.

Farnsworth KD, Niklas KJ, 1995. Theories of Optimization, Form and Function in Branching Architecture in Plants. Functional Ecology 9:355-363.

Gavrilets S, Hayashi TI, 2005. Speciation and sexual conflict. Evol Ecol 19:167-198.

González-Santoyo I, Córdoba-Aguilar A, 2012. Phenoloxidase: a key component of the insect immune system. Entomologia Experimentalis et Applicata 142:1-16.

Hodjat S, 1970. Effects of crowding on colour, size and larval activity of Spodoptera littoralis (Lepidoptera: Noctuidae). Entomologia experimentalis et applicata 13:97-106.

Iwanaga S, Lee B-L, 2005. Recent advances in the innate immunity of invertebrate animals. BMB Reports 38:128-150.

Kristensen CO, 1994. Investigations on the natural mortality of eggs and larvae of the large white *Pieris brassicae* (L.) (Lep., Pieridae). Journal of Applied Entomology 117:92-98.

Lang A, Filser J, Henschel JR, 1999. Predation by ground beetles and wolf spiders on herbivorous insects in a maize crop. Agriculture, ecosystems & environment 72:189-199.

Lee KP, Wilson K, 2006. Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait. Ecological Entomology 31:196-205.

Mirth CK, Riddiford LM, 2007. Size assessment and growth control: how adult size is determined in insects. Bioessays 29:344-355.

Moczek A, Emlen D, 1999. Proximate determination of male horn dimorphism in the beetle *Ontophagus taurus* (Coleoptera: Scarabaeidae). Journal of Evolutionary Biology 12:27-37.

Moczek AP, Emlen DJ, 2000. Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? Animal behaviour 59:459-466.

Nijhout HF, 2003. The control of body size in insects. Developmental Biology 261:1-9.

Reichle D, 1968. Relation of Body Size to Food Intake, Oxygen Consumption, and Trace Element Metabolism in Forest Floor Arthropods. Ecology 49:538-542.

Schmid-Hempel P, 2005. Evolutionary ecology of insect immune defenses. Annu Rev Entomol 50:529-551.

Strand MR, 2008. The insect cellular immune response. Insect Science 15:1-14.

Taborsky M, Brockmann HJ, 2010. Alternative repro-

ductive tactics and life history phenotypes. In: Kappeler P, editor. Animal Behaviour: Evolution and Mechanisms: Springer Berlin Heidelberg. p. 537-586.

Talloen W, Dyck HV, Lens L, 2004. The cost of melanization: Butterfly wing coloration underenvironmental stress. Evolution 58:360-366.

van Doorn GS, Dieckmann U, Weissing FJ, 2004. Sympatric speciation by sexual selection: a critical reevaluation. The American Naturalist 163:709-725.

Wittkopp PJ, Stewart EE, Arnold LL, Neidert AH, Haerum BK, Thompson EM, Akhras S, Smith-Winberry G, Shefner L, 2009. Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila*. Science 326:540-544.

Wooster D, 1994. Predator impacts on stream benthic prey. Oecologia 99:7-15.

ACKNOWLEDGMENTS

Well well... Throughout these years an immense number of people has provided me help and support so I will do my best to recognize their company from far and near along this wonderful journey that was the PhD. I won't include last names cause I know everyone referred here will know that I am talking to you ;).

I will like to start thanking my family. In spite of the distance, they have always supported my work and my life over here. Gracias Ma, Pa, Belén! I miss them a lot but their good humor has always kept me up over here, across the 12.000 km that separate us physically, but not sentimentally. I would also like to thank Christina, because she has become a really important person in my life, hearing my problems, my presentations and helping me on the different stages along this long road.

I have to say that the lab members became a second family to me, assuming all kind of crazy fun roles. I would like to first thank Wolf Blanckenhorn, because if it wasn't for him all this would have just been a dream, but his help and support made it reality. Being part of the Blanckenhorn lab introduced me to people who helped me with experiments, happy hours, not so happy busy hours and fun times outside of the lab. Thanks Valérian, Patrick, Alex, Natalia, Martin, Julian, Jeannine, Athene, Maja, Hayat, Anders, Hiroshi, Mariana and Daniel for adding each one color to my life over here. Also, recently we incorporated Stefan, Jhoniel, Sonja and Lea who although are technically part of a "different lab", the real deal is that we all work with flies and share the passion for smelly fly substrates.

Among this institute there is people distributed everywhere to whom I want to thank because they have accompanied me as well: Chantal, Lara, Sarah, Sam, Tina, Nino, Koen, Aurélie, Vanessa, Frank, Rich, Wilfredo, Alejandra, Joel, Ali, Marco, Christina, Gaurav and Hedwig. But the friendships extend even further than one thinks, in other floors and labs I would like to thank Jacqueline, Pirmin, Philipp, Judith, Beni, Rassim, Reini, Nina, Alex, Max, Manu, Denise, Jan, Andri, Andreas, Jobran, Ramona, Cini, Julia, Luis and Bruce.

Also many professors and researchers have provided academic support. Thank you members of the committee members: Josh and Barbara. Their commentaries during our meetings really helped me lead this work in the right direction. However, many other professors have help with comments, talks and suggestions, so thank you: Owen, Dennis, Erik and Barbara.

A very special thanks to the wonderful field helpers that accompanied me in Italy. Muchas gracias Tano y Francés! Los espero para seguir juntando moscas!

I would also like to thank the administrative staff, specially Isabelle because she has always been there doing all the paper work, allowing this thesis to run smoothly as a Swiss train.

To all of you, thank you! Thank you! If I could write a thank you thesis I would. But since time is pressing and you all know how much I care about you, I hope that the humble inclusion of your name in my thesis will make you feel included as part of my life. It think that the important thing is not a written name, but a shared feeling and experience.

It was a pleasure getting to know you all! Thanks again for everything! I take great memories with me!

JP

P.S.: If I happened to forget someone, it was not my intention, but I only have a couple of days left to wrap everything up :).

CURRICULUM

PERSONAL DETAILS

Name: Juan Pablo Busso

Nationality: Argentinian and Italian

Home address: Stampfenbachstrasse 151, CH-8050 Zürich

Date of Birth: 28 February 1986

Phone numbers: (+41) 79 137 37 51

E-mail: juanpablo.busso@ieu.uzh.ch

EDUCATION:

2013-current PhD Student in Ecology and Evolutionary Biology. *Institute of Evolutionary Biology and Environmental Studies. University of Zürich*.

Project: "Interplay of natural and sexual selection on the evolution of intraspecific discrete phenotypes in *Sepsis thoracica*" with Prof. Dr. Wolf U. Blanckenhorn.

2013 Master's degree in Ecology and Evolutionary Biology. *University of Michigan*, United States. GPA: 8.277 (on a scale of 0 to 9).

Project: "Variation of female preference influences the maintenance of phenotypic diversity" with Dr. Alison Davis Rabosky and Prof. Dr. Earl Werner.

2011 - 2013 Master Student in Ecology and Evolutionary Biology. *University of Michigan*, United States.

2010 Biology degree, College of Exact, Physical and Natural Sciences, *National University of Córdoba*, Argentina. GPA: 9.71 (on a scale of 0 to 10).

2005 - 2010 Student of Biological Sciences, College of Exact, Physical and Natural Sciences, *National University of Córdoba*, Argentina.

2004 Senior Year in Raymond High School. Raymond, Alberta, Canada.

2001 - 2003 High School with orientation in Humanities and Social Sciences, *Nuestra Señora de las Mercedes Institute*. Unquillo, Córdoba, Argentina. GPA: 9.80 (on a scale of 0 to 10).

1998 - 2000 Middle School, *Nuestra Señora de las Mercedes Institute*, Unquillo, Córdoba, Argentina. GPA:

9.54 (on a scale of 0 to 10).

OTHER COURSES:

2010 Introduction to Electronics. *Laboratory of Digital Electronics.* Córdoba, Argentina.

2009 Redaction of Advanced and Scientific Texts. *Center of Advance Studies of the National University of Córdoba*. Argentina.

2009 Physiology, biochemistry, cellular and molecular biology of insects of Economic and Public Health importance. *College of Chemistry*, National University of Córdoba. Argentina.

2008 Urban Arboriculture: Knowledge and Management of Public Trees. *Society of Biologists of the Province of Córdoba*. Argentina.

2008 Introduction to the flora and fauna of "Serranos" environments. *Argentinian Ecosystems*. Córdoba, Argentina.

TEACHING EXPERIENCE:

2016 Course Assistant for Ecology (BIO 329). Universität Zürich.

2015 Course Assistant for Principles of Evolution (BIO 352). Universität Zürich.

2015 Course Assistant for Ecology (BIO 329). Universität Zürich.

2015 Course Assistant for Biology of Reproduction (BIO 361). Universität Zürich.

2014 Course Assistant for Biodiversität: Wirbellose, Wirbeltiere, Pilze (BIO 121). Universität Zürich.

2014 Course Assistant for Biology of Reproduction (BIO 361). Universität Zürich.

2013 Graduate Student Instructor for Introduction to Biology Lab (BIO 173). Department of Biology. University of Michigan.

2012 Graduate Student Instructor for Introduction to Biology Lab (BIO 173). Department of Biology. University of Michigan.

2011 Graduate Student Instructor for Introductory Biology: Ecology and Evolution (BIO 171). Department of Biology. University of Michigan.

2010 Teaching Assistantship in the Animal Diversity I Department. College of Exact, Physical and Natural Sciences, *National University of Córdoba*, Argentina.

RESEARCH EXPERIENCE:

2013-current PhD Student in Ecology and Evolutionary Biology. *Institute of Evolutionary Biology and Environmental Studies. University of Zürich*.

Project: "Selection on trade-offs of the dimorphic black scavenger fly *Sepsis thoracica*" with Prof. Dr. Wolf U. Blanckenhorn.

2012 - 2013 Master thesis titled: "Variation in female preference influences the maintenance of phenotypic diversity". University of Michigan, United States.

2010 Thesis titled: "Factors regulating the feeding preference of the common woodlouse (*Armadillidium vulgare*)". It was conducted in the National Institute of Agricultural Technology (INTA), Argentina.

2009-2010 Research Assistantship in the Animal Diversity II Department. College of Exact, Physical and Natural Sciences, *National University of Córdoba*, Argentina.

LANGUAGES:

SPANISH: Mother tongue.

ENGLISH: Advanced Level. English House Institute (10 years of studies). Unquillo, Córdoba, Argentina.

Rotary Youth Exchange in Canada for a year (2004).

Masters Studies at the University of Michigan.

FRENCH: B1. Sprachwelten Sprachschule. Zürich, Switzerland.

GERMAN: C1. Bellingua Institute. Zürich, Switzerland.

PROGRAMMING:

Advanced Level in language ActionScript 3.0.

Advanced Level in R (Statistics programing language).

HONORS, AWARDS OR ACCOMPLISHMENTS:

2015 Claraz-Donation provided by the Georges and Antoine Claraz Stiftung for the project "The role of developmental temperature for predation vulnerability in the black scavenger fly Sepsis thoracica". (CHF 2'300)

2015 Second Prize for Poster in the conference Biology 2015 in Dübendorf, Switzerland.

2012 Block Grant Fellowship provided by the Rackham School of Graduate Studies for Research Project entitled "Female preference in *Maevia inclemens* and its influence on alternative reproductive tactics". (USD 841 to cover research expenses)

2012 Rackham Graduate Student Research Grant provided by the Rackham School of Graduate Studies for Research Project entitled "Female preference in *Maevia inclemens* and its influence on alternative reproductive tactics". (USD 1'500 to cover research expenses)

2010 Highest GPA in the College of Exact, Physical and Natural Sciences Award granted by the *Society of Biologist of the Province of Córdoba*.

2010 Highest GPA in the College of Exact, Physical and Natural Sciences Award granted by the *Graduates Association of College of Exact, Physical and Natural Sciences*.

2010 Best Research Award in the *Health Sciences Students Scientific and Academic Conference* (Jornadas Científicas y Académicas de Estudiantes de Ciencias de la Salud) for the work entitled "Análisis de la contaminación en los dos cauces principales que atraviesan la localidad de Unquillo empleando macroinvertebrados bentónicos como bioindicadores".

2009 Highest GPA in the College of Exact, Physical and Natural Sciences Award granted by the *Provincial Society of Engineers*.

2009 Standard-bearer of *College of Exact, Physical and Natural Sciences.* National University of Córdoba.

2008 Standard-escort of *College of Exact, Physical and Natural Sciences.* National University of Córdoba.

2003 Academic Excellence Award awarded by *Banco Roela*.

SERVICE AND OUTREACH

2009 Presentation of the work entitled "Análisis de la contaminación en los dos cauces principales que atraviesan la localidad de Unquillo empleando macroinvertebrados bentónicos como bioindicadores" to Water and Public Services Cooperative of Unquillo, Córdoba. The presentation introduced contamination related problems detected in the rivers and alternative solutions to address them.

MANUSCRIPTS

2017 **Busso, Juan Pablo**, Blanckenhorn, W. U. And Gonzáles-Tokman, D. Healthier or bigger? Trade-off mediating male dimorphism in the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae). *Ecological Entomology*. doi:10.1111/een.12413

2017 **Busso, Juan Pablo** and Blanckenhorn, Wolf. Size trumps coloration: Predation selection on dimorphic males of the scavenger fly *Sepsis thoracica*. *Behavioral Ecology* (*under review*).

2017 **Busso, Juan Pablo** Climatic factors shaping plastic trade-offs in the polyphenic black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae). *Journal of Biogeography* (*under review*).

2017 **Busso, Juan Pablo**; Trumper, Eduardo and María José Martinez. "Soybean or detritus? Factors affecting the feeding preference of the emergent pest *Armadillidium vulgare* (Isopoda: Oniscidea)" *Journal of Pest Science* (*under review*).

2016 **Busso, Juan Pablo**, and Alison R. Davis Rabosky. "Disruptive selection on male reproductive polymorphism in a jumping spider, *Maevia inclemens*." *Animal Behaviour* 120 (2016): 1-10.